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GROWTH

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THE TRYPTOPHANE, LEUCINE, AND LYSINE CONTENT OF THE CHICK AND MOUSE EMBRYO DURING DEVELOPMENT[1]

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INTRODUCTION

In 1952, Albanese (1) pointed out that the optimum dietary amino acid pattern for maintenance of human adults is different from that for growth of infants. Steffee *et al.* found that the amino acid pattern for maintenance of rats was different from that for repletion of protein-depleted rats (33). Both groups found that a higher proportion of lysine and leucine to tryptophane is required during increased protein synthesis.

More recently, Albanese *et al.* (2) have emphasized that an increased lysine to tryptophane (L/T) ratio in the diet is necessary for optimum growth in infants. This claim is contested by Holt and his co-workers (11), who assert that infants have no need of an increased L/T ratio in the diet.

Munks *et al.* (16) and Mitchell (15) have proposed that the amino acid analysis of a whole organism, or major tissue (muscle, for example) thereof, is a reliable method for estimating the dietary amino acid requirements of this organism. Williams *et al.* (37) tested this idea by analyzing rat, chick, and pig carcasses for amino acid content at various post-natal ages. They found that the calculated requirements based on carcass analysis are very similar to those which have been determined by nutritional experiments, if adequate consideration is given to the polyfunction, or sparing action of certain amino acids. In addition, their data show that the ratios of lysine and of leucine to tryptophane increase during post-embryonic development of both

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the rat and chick, while they decrease slightly and irregularly in the pig. The experimental results of Albanese and of Steffee *et al.* had suggested that there would be a decrease as the animal approached adulthood.

In a preliminary investigation carried out in this laboratory (30), the ratios of lysine and leucine to tryptophane were found to be higher in embryonic and cancerous tissue than in resting or regenerating liver. This finding reaffirmed the similarity of embryonic and neoplastic growth as contrasted to regenerative growth (7, p. 430 *et seq.*). The results, however, shed little light on the significance of the ratio differences observed by the Albanese, Steffee, and Williams groups. In order to confirm and, if possible, clarify our earlier findings and those of the other investigators, the present series of experiments was undertaken.

This paper deals with the results of the analyses of the chick embryo from the 7th to the 18th day of incubation, and of the mouse embryo from the 12th to the 20th day of gestation. The fresh weight, and the amounts of dry solid, protein, and protein-bound tryptophane, lysine, and leucine have been determined.

Other investigators have reported similar and more extensive analyses. Since the publication of Needham's "Chemical Embryology" (17), Schenck (27), Calvery (4), and more recently Rupe and Farmer (26), have investigated the amino acid composition of the chick during embryonic growth. Price *et al.* (20) and Williams *et al.* (37) have studied the changes subsequent to hatching. Gonzales and Awapara (6) determined taurine, Robinson (24, 25) the total protein and nucleoprotein content of chick muscle, and Szepsenwol *et al.* (35), the nitrogen concentrations of the chick embryo during growth.

Very little work has been done on the gross composition of the mammalian embryo since Wilkerson and Gortner (36) investigated the amino acid metabolism of the pig embryo. In contrast, excellent quantitative studies have been made of invertebrate embryo amino acid metabolism. The sea urchin embryo has been investigated by Gustafson and Hjelte (9), and by Kavanau (12). *Bombyx mori* has been studied by Ricceri (23).

The more general aspects of embryology have been recently reviewed by Needham (18), Newth (19), and Spratt (32).

EXPERIMENTAL

In the study of the chick embryo, two different strains were used, *viz.* Ketay X-99 (Barred Rock *vs.* White Rock) and Ketay White Leghorn (obtained from Ketay Hatcheries, Huntington, Long Island, New York). The chick embryos were incubated and separated as described by Reddy *et al.* (22), and stored at —20° C. until analyzed.

Pregnant female Swiss mice were obtained from Rockland Farms (New City, New York). The females were decapitated, and the embryos removed and handled according to the technique described above (22). The age of the mouse embryos was estimated from the body weight and the crown-rump length, by comparison with the data of Grüneberg (8), MacDowell *et al.* (13), and Reddy and Cerecedo (21).

The tissues were homogenized, and aliquots of the homogenates were taken for the determination of dry solid weight, and for purification using Schneider's procedure (29). Following extraction of the nucleic acids, the washed tissues were brought to constant weight *in vacuo* over H_2SO_4. Portions of the dry material were taken for determination of the 105° dry weight, lysine, leucine, and tryptophane.

Protein determination.—The "Schneider residue," corrected to the 105° dry weight, is taken to be pure protein.

Lysine and leucine determination.—The assay was carried out on the hydrolyzates obtained by heating protein with 3N HCl (0.2 ml. per mg.) for 8 hours at 15 pounds pressure in an autoclave. *Streptococcus faecalis* ATCC 9790 was used for the assay of both amino acids in the chick, and for leucine assay in the mouse. *Leuconostoc mesenteroides* P-60 was used for lysine assay in the mouse. In the assay of leucine, a high-citrate, low-acetate medium gave excellent results. The methods of Henderson and Snell (10) and Stokes *et al.* (34) formed the basis of the assay procedure employed. All microbiological determinations for a given strain or species of embryo were carried out simultaneously, at four levels in duplicate.

Tryptophane determination.—In the X-99 chick embryo, tryptophane was determined using *S. faecalis*. The protein was hydrolyzed with 5N NaOH (0.12 ml. per mg.) for 8 hours at 15 pounds pressure.

Because basic hydrolysis introduces a number of variables (racemization, destruction, adsorption on silicate), the chemical method of Spies and Chambers (31) was used for the determination of tryptophane in both the White Leghorn chick and Swiss mouse embryos.

Results

In Table I we present the data on the number of embryos and the number of groups of embryos analyzed, and the analytical data for the amino acids that were determined.

TABLE I

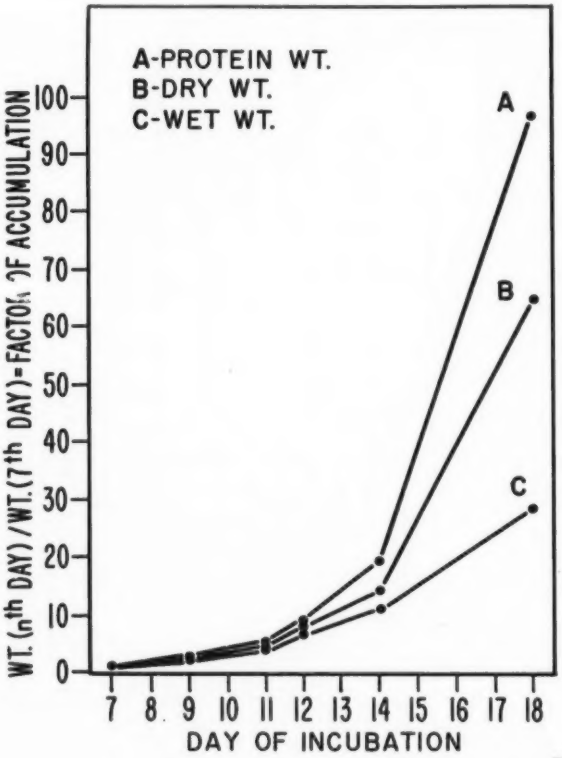
Day of Growth	No. of Embryos	No. of Groups	Amino Acid Residues as Per Cent Protein		
			Tryptophane	Lysine	Leucine
Swiss Mouse Embryo:					
12	17	2	0.76 ± .01*	4.8**	5.9 ± .0
15	8	2	0.81**	5.05 ± .05	5.6 ± .1
17	10	2	0.80 ± .035	5.2 ± .5	5.8 ± .0
19	10	2	0.81 ± .075	5.6 ± .5	6.1**
20	7	2	0.91 ± .01	5.0 ± .1	6.0**
White Leghorn Chick Embryo:					
7	20	1	1.29	7.5	7.5
9	10	1	1.31	7.1	7.8
11	10	2	1.27 ± .04	7.6 ± .1	8.0 ± .1
12A***	4	1	1.29	7.5	7.4
12B	4	1	1.19	8.6	7.5
12 (Av.)	8	2	1.24 ± .05	8.1 ± .5	7.05 ± .05
14	6	2	1.36 ± .09	8.0 ± .1	7.8 ± .2
18	3	3	0.98 ± .05	6.0 ± .2	7.5 ± .1
X-99 Chick Embryo:					
8	16	3	0.85 ± .02	7.7 ± .5	6.9 ± .0
9	7	1	0.98	6.5	6.9
11	4	2	0.85**	7.45 ± .25	7.35 ± .15
12	2	1	0.97	7.7	6.8
13	3	3	0.82 ± .06	10.2 ± .2	6.5 ± .4
15	2	2	0.83 ± .095	7.2 ± .1	7.45 ± .05
18	6	3	0.68 ± .08	5.2 ± .4	7.6 ± .3

* Means ± Standard deviation.
** One sample analyzed.
*** See graph of lysine curve.

Data on the chick embryo.—Figure Ia shows the comparative build-up of the wet, dry solid, and protein weights. The dry weight is amassed more rapidly than wet weight, reflecting the decreasing water concentration of the embryo. The protein increases more rapidly than the total dry solids, indicating a decrease in the relative non-protein (lipid?) content during growth.

In Figure Ib-A it is seen that the most rapid increase in the protein content of the embryo occurs just before hatching. The protein fraction of the dry solids (Figure Ib-B) continually increases during incubation. The greatest change is at the 12-13th day.

Figure II-A shows the level of protein-bound tryptophane in both



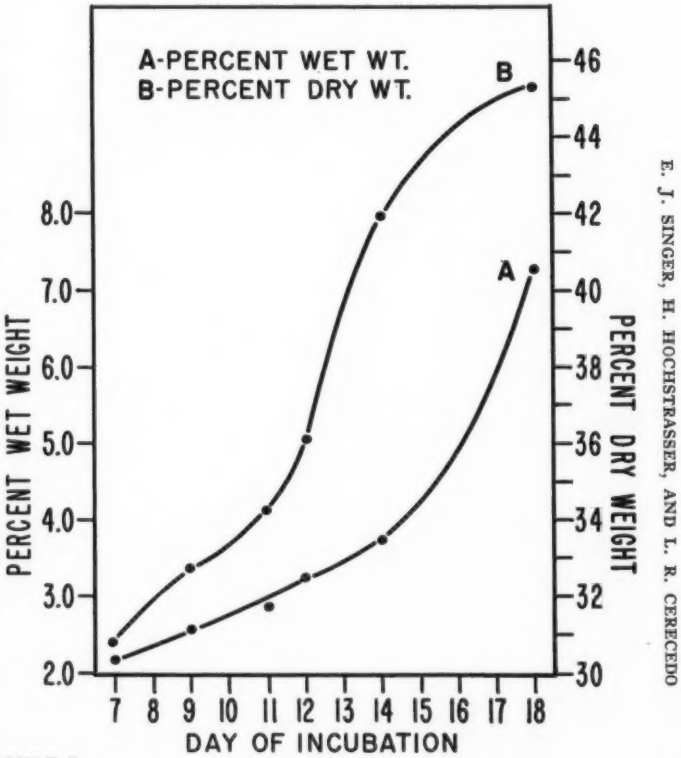


FIGURE Ia.
Accumulation of protein (A), dry (B), and wet (C) weight during growth of the White Leghorn chick embryo.
FIGURE Ib.
Variation in the protein content of the White Leghorn chick embryo during growth. (A) Protein as percent of wet weight. (B) Protein as percent of dry weight.

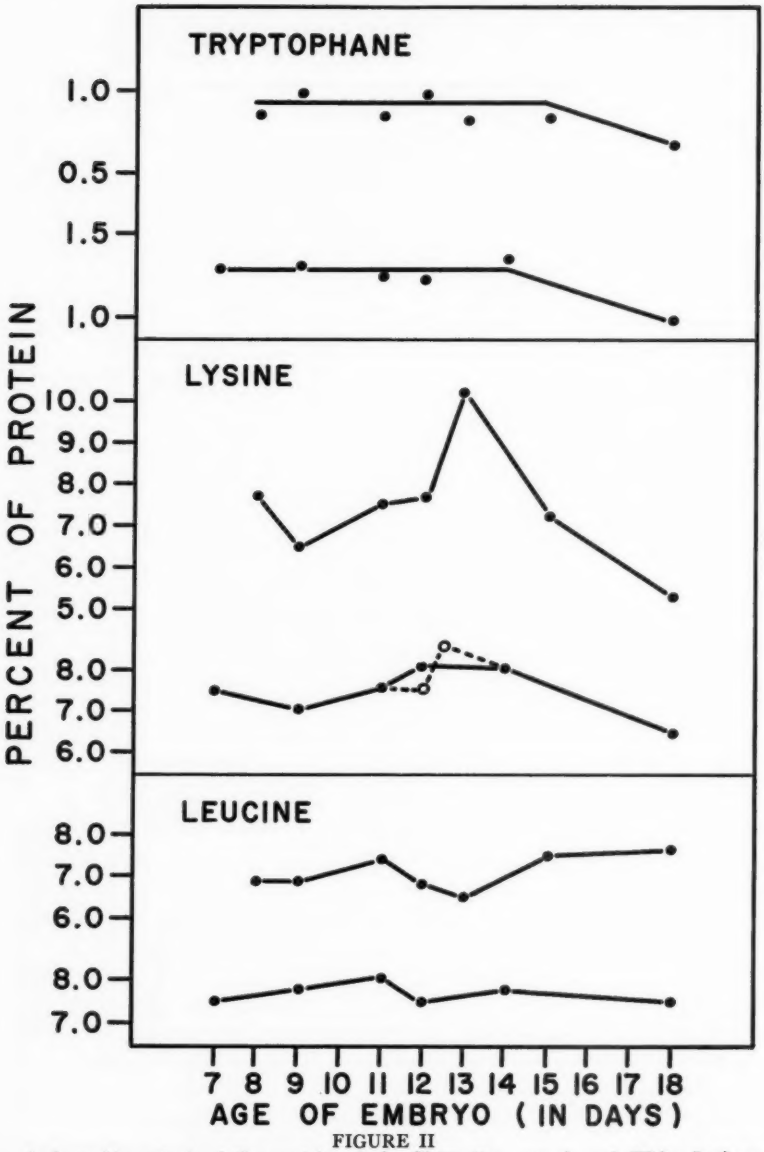


FIGURE II

Amino-acid content of the protein of the X-99 (top curve) and White Leghorn (bottom curve) chick embryo during growth. (A) The tryptophane content. (B) The lysine content. The dashed line (—o—) shows the shape of the curve when the second group of White Leghorn embryos sacrificed on the 12th day is taken to be 12.5 days of age. (C) The leucine content.

the X-99 and White Leghorn chick embryos during incubation. The tryptophane initially remains fairly constant, after which it drops to a minimum on the 18th day.

In both strains, there is a decrease in the lysine content (Figure II-B) to the 9th day, an increase to a peak on the 12-13th day, and then a drop to a minimum value just before hatching. All these changes are more pronounced in the X-99 embryo.

TABLE II
The Ratio of Leucine and Lysine to Tryptophane During Embryonic Development of the White Leghorn Chick.

(Tryptophane is taken as equal to unity)

Day of Incubation	Tryptophane :	Lysine :	Leucine
7	1.0	5.8	5.8
9	1.0	5.4	6.0
11	1.0	6.0	6.3
12	1.0	6.5	6.0
14	1.0	5.9	5.7
18	1.0	6.6	7.6

The changes in leucine content (Figure II-C) are similar in both strains. There is a maximum at the 11th day, and a minimum at the 12-13th day, followed by a levelling-off.

Data on the mouse embryo.—Figure IIIa shows the relative accumulation of the fresh and protein weights during embryonic growth in the mouse. This is similar to that observed in the chick. As in the chick, there is a gradual increase in the protein as percent of wet weight (Figure IIIb) until just before the end of embryonic life, when there is a sharp increase.

The tryptophane content (Figure IV-A) of the mouse embryo increases from the 12th to the 15th day, levels off, and then shows a sharp rise to a higher value on the 20th day.

The lysine content of the protein (Figure IV-B) increases gradually until the 19th day, after which there is a sudden decrease.

The leucine content (Figure IV-C) remains fairly constant from the 12th to the 20th day of gestation, exhibiting a slight minimum at the 15th day.

The *tryptophane:lysine:leucine ratio.*—In Tables II and III are given the ratios of lysine and leucine to tryptophane at different periods of growth in each of the two species studied. In the chick (Table II), the ratio values increase irregularly, leucine and lysine

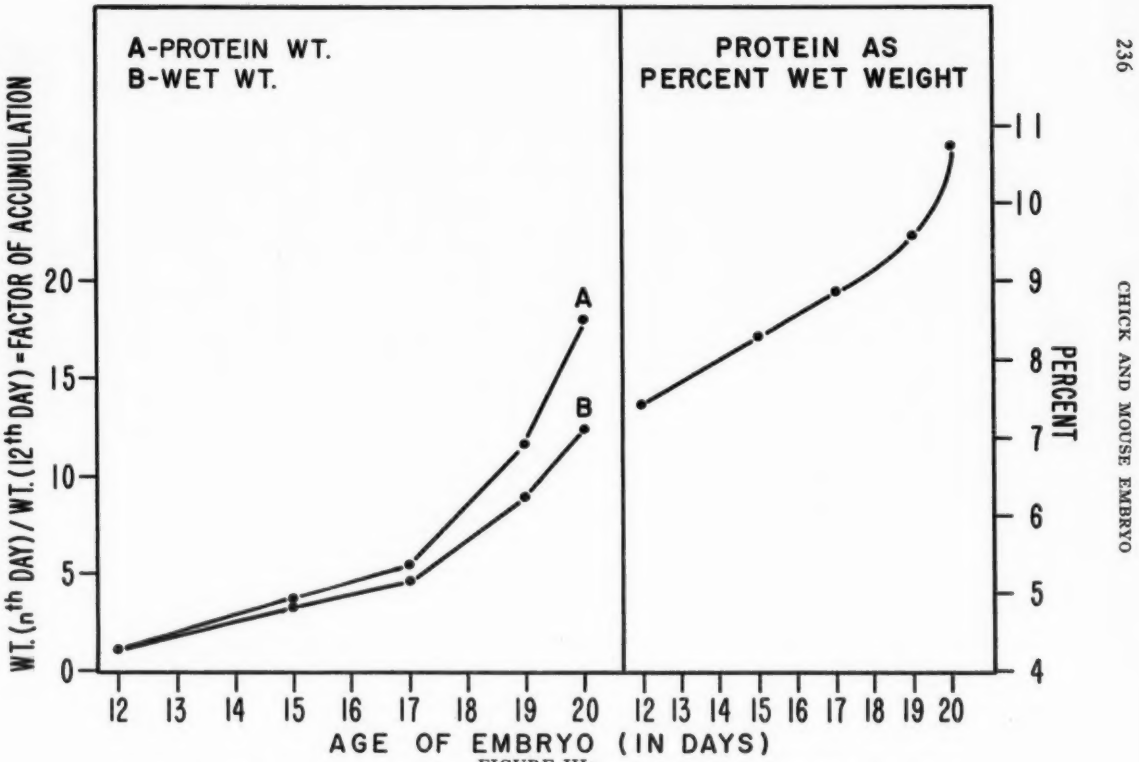


FIGURE IIIa.
Accumulation of protein (A) and wet (B) weight during embryonic growth of the Swiss mouse.
FIGURE IIIb.
Variation in the protein content of the Swiss mouse embryo during growth, expressed as percent of fresh weight.

reaching maximal values on the 11th and 12th day, respectively. There is then a decrease, followed by an increase to maximal values on the 18th day.

In the mouse (Table III), the ratio values show a gradual increase from the 15th to the 19th day, dropping to minima on the 20th day.

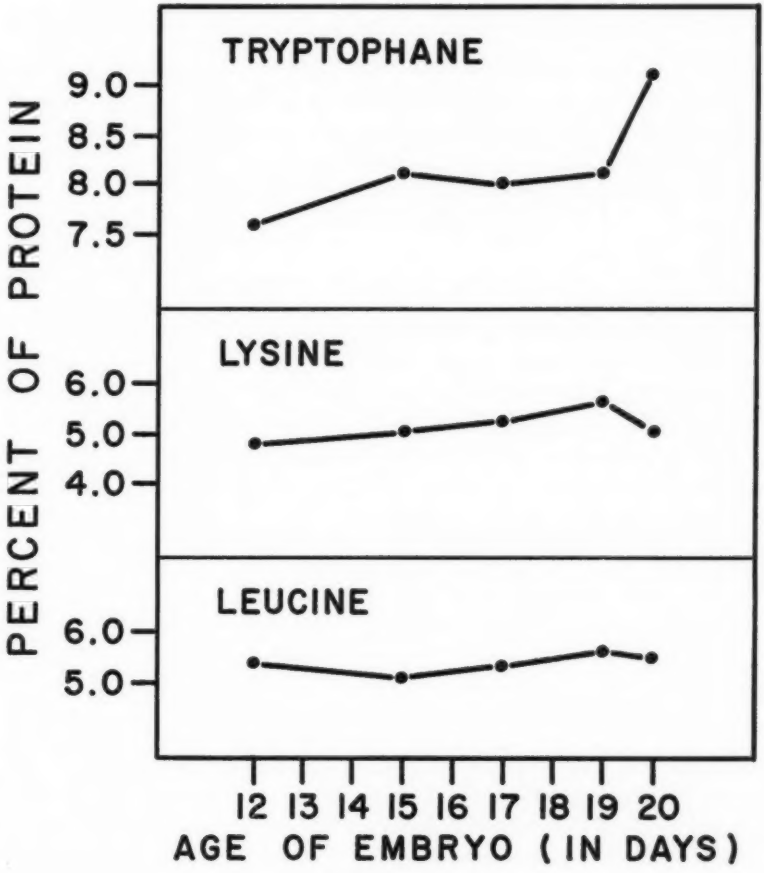


FIGURE IV

Amino-acid content of the protein of the Swiss mouse embryo during growth. (A) The tryptophane content. (B) The lysine content. (C) The leucine content.

TABLE III
The Ratio of Leucine and Lysine to Tryptophane During Embryonic Development of the Swiss Mouse.

Day of Gestation	Tryptophane :	Lysine :	Leucine
12	1.0	6.3	7.8
15	1.0	6.2	6.9
17	1.0	6.5	7.3
19	1.0	6.9	7.5
20	1.0	5.5	6.6

Discussion

Our data (Figures Ia and Ib-B) indicate a continuous increase in the protein of the chick embryo when expressed as percent of dry weight. The data given by Needham (16, Vol. II, p. 913) show a similar trend until the 15-16th days, after which the protein content decreases. The following should be noted: (a) Our protein values increase from about 31 to 45% from the 7th to the 18th day, Needham's values from 55 to 64% during the same period. It may be seen that our figures are lower, and increase more rapidly than Needham's, during the development of the chick. (b) Needham observed a steady decrease in the protein values after the 16th day. Although we did find a decrease in the slope of the protein curve (Figure Ib-B) after the 14th day, the values continued to increase through the 18th day.

We have found a fairly sharp decrease in the tryptophane content of the chick embryo just before hatching. Price *et al.* (20) observed a further decrease in the tryptophane from the 1st to the 4th week after hatching. Williams *et al.* (37) observed a similar drop from the 1st day to the 10th week of post-embryonic life. In contrast to our results, both Schenck (27) and Calvery (4) found a progressive increase in the tryptophane content of the chick embryo during development. The discrepancies in the tryptophane values may be the result of the method of estimation. The method of May and Rose (14), which was used by Schenck, makes no provision for the use of protein "blanks." Spies and Chambers found the use of such blanks to be necessary. Calvery used the method of Folin and Ciocalteu (5), which has been criticized for its non-specificity by Block (3, p. 130), on the basis of the investigations of Schild and Enders (28).

Our lysine data for the chick show a peak on the 12-13th day; Schenck's data evidenced no peak, and Calvery found a minimum value in the region of the 12th to the 15th day. With reference to

data in earlier papers of Calvery, Wilkerson and Gortner state (36, p. 155) that ". . . the . . . lysine results were admittedly undependable."

Neither Schenck nor Calvery determined leucine. Although Rupe and Farmer (26) determined this amino acid (and thirteen others), their data are expressed on a fresh weight basis, and do not indicate the variation in the amino acid content of the chick protein as such.

Since we know of no other growth studies in the mouse embryo, we shall compare our data to those of Wilkerson and Gortner (36). They found a gradual increase in the lysine content of the pig embryo. Our data also show a gradual increase in the lysine content of the mouse embryo. There is, however, a sharp decrease in the lysine of the mouse just before birth. While the lysine values of Wilkerson and Gortner show no significant decrease, they do level off to a fairly constant value near term. Williams *et al.* (37) did, however, find a tendency for the lysine to decrease during the post-natal development of the pig.

Kavanau (12) fractionated the protein of the sea urchin embryo and found a series of cyclic changes in the amino acid content of each of the two fractions. The successive maxima and minima we have observed, particularly in the chick embryo, may reflect this type of cyclic variation.

The interpretation of the ratio data is difficult. The changes in the amino acid patterns are different in the chick and the mouse. The patterns themselves, nevertheless, are fairly similar until just before the end of embryonic life. At this time, the ratio of lysine and leucine to tryptophane is highest in the chick, and lowest in the mouse.

In the light of the nutritional findings mentioned earlier (see Introduction), a high proportion could reflect the rapid protein synthesis just before term (shown in Figures Ib-A and IIIb-A). A low proportion could be interpreted to signal the end of embryonic life. In the absence of other data, however, no explanation of the ratio changes will be attempted.

Summary

Chemical changes occurring in the chick and mouse embryo during development have been studied. The fresh weight, dry solid, protein, and protein-bound tryptophane, lysine, and leucine have been determined.

In both species, there is a sharp increase in protein synthesis at the end of embryonic development. At this time, the protein-bound tryptophane decreases in the chick, and increases in the mouse. It is otherwise fairly constant. In both the chick and mouse embryo, the lysine content increases, reaches a peak, and then drops fairly sharply at the end of the period investigated. In both species, there is little change in the leucine content of the protein during embryonic development.

The proportion of lysine and leucine to tryptophane is highest in the chick embryo, and lowest in the mouse embryo, just preceding hatching and birth, respectively. No explanation is offered for this observed result.

References

1. Albanese, A. A. 1952. The effects of amino acid deficiencies in man. *Am. J. Clin. Nutrition,* **1**, 44-51.
2. Albanese, A. A., Higgons, R. A., Hyde, G. M., & Orto, L. 1956. Lysine and tryptophan content of proteins and their utilization for human growth. *Am. J. Clin. Nutrition,* **4**, 161-168.
3. Block, R. J., & Bolling, D. 1951. The Amino Acid Composition of Proteins and Foods. Analytical Methods and Results. 2nd Ed. Springfield: Thomas. Pp. 576.
4. Calvery, H. O. 1932. Some chemical investigations of embryonic metabolism. VI. Studies of some of the amino acids of the yolk, white, embryo, and shell membranes during development of the hen's egg. *J. Biol. Chem.,* **95**, 297-300.
5. Folin, O., & Ciocalteu, V. 1927. On tyrosine and tryptophan determinations in proteins. *J. Biol. Chem.,* **73**, 627-650.
6. Gonzales, F., & Awapara, J. 1955. The taurine concentration of developing chick embryo. *Exptl. Cell Research,* **9**, 353-355.
7. Greenstein, J. P. 1954. Biochemistry of Cancer. 2nd Ed. New York: Academic Press. Pp. 653.
8. Grüneberg, H. 1943. The development of some external features in mouse embryos. *J. Hered.,* **34**, 88-92.
9. Gustafson, T., & Hjelte, M. B. 1951. The amino acid metabolism of the developing sea-urchin egg. *Exptl. Cell Research,* **2**, 474-490.
10. Henderson, L. M., & Snell, E. E. 1948. A uniform medium for determination of amino acids with various microorganisms. *J. Biol. Chem.,* **172**, 15-29.
11. Holt, L. E., Jr., & Snyderman, S. E. 1956. The amino acid requirements of children. Some Aspects of Amino Acid Supplementation, 60-68. New Brunswick: Rutgers University Press. Pp. 85.
12. Kavanau, J. L. 1954. Amino acid metabolism in the early development of the sea urchin *Paracentrotus lividus. Exptl. Cell Research,* **7**, 530-557.
13. MacDowell, E. C., Allen, E., & MacDowell, C. G. 1927. The prenatal growth of the mouse. *J. Gen. Physiol.,* **11**, 57-70.
14. May, C. E., & Rose, E. R. 1922. The tryptophane content of some proteins. *J. Biol. Chem.,* **54**, 213-216.
15. Mitchell, H. H. 1950. Some species and age differences in amino acid requirements. Protein and Amino Acid Requirements of Mammals, 13-18. New York: Academic Press. Pp. 155.

16. MUNKS, B., ROBINSON, A., BEACH, E. F., & WILLIAMS, H. H. 1945. Amino acids in the production of chicken egg and muscle. *Poultry Sci.*, **24**, 459-464.

17. NEEDHAM, J. 1931. Chemical Embryology. London: Cambridge University Press. 3 Vol., pp. 2021.

18. ————. 1955. Developmental physiology. *Ann. Rev. Physiol.*, **17**, 37-60.

19. NEWTH, D. R. (Editor). 1954. Recent advances in animal morphogenesis; Conference on Embryology and Experimental Morphology, London, 1954. *Nature*, **173**, 517-520.

20. PRICE, W. A., JR., TAYLOR, M. W., & RUSSELL, W. C. 1953. The retention of essential amino acids by the growing chick. *J. Nutrition*, **51**, 413-422.

21. REDDY, D. V. N., & CERECEDO, L. R. 1951. Nucleic acid changes in the mouse during gestation. *J. Biol. Chem.*, **192**, 57-61.

22. ————, LOMBARDO, M. E., & CERECEDO, L. R. 1952. Nucleic acid changes during the development of the chick embryo. *J. Biol. Chem.*, **198**, 267-270.

23. RICCERI, G. 1952. Quantitative variations of individual amino acids in the eggs of *Bombyx mori* during embryonic development. *Arch. sci. biol.*, **36**, 376-393. Ref., *Chem. Abstr.*, **48**, 2934b (1954).

24. ROBINSON, D. S. 1952. Changes in the protein composition of chick muscle during development. *Biochem. J.*, **52**, 621-628.

25. ————. 1952. Changes in the nucleoprotein content of chick muscle during development. *Biochem. J.*, **52**, 628-633.

26. RUPE, C. O., & FARMER, C. J. 1955. Amino acid studies in the transformation of proteins of the hen's egg to tissue proteins during incubation. *J. Biol. Chem.*, **213**, 899-906.

27. SCHENCK, E. G. 1932. I. Untersuchungen über das Verhalten der Eiweissstoffe bei der Bebrütung des Hühnereies. *Z. physiol. Chem.*, **211**, 111-153.

28. SCHILD, E., & ENDERS, C. 1936. Zur Spezifität des Phenolreagens nach Folin und Ciocalteu für die Tryptophanbestimmung. *Biochem. Ztschr.*, **286**, 220-224.

29. SCHNEIDER, W. C. 1945. Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. *J. Biol. Chem.*, **161**, 293-303.

30. SINGER, E. J., DARMSTADT, R. A., & CERECEDO, L. R. 1954. Tissue proteins during growth; the tryptophane, lysine and leucine content. Paper presented at A.C.S. Meeting-in-Miniature, February 12, Hunter College, New York.

31. SPIES, J. R., & CHAMBERS, D. C. 1949. Chemical determination of tryptophan in proteins. *Anal. Chem.*, **21**, 1249-1266.

32. SPRATT, N. T., JR. 1954. Physiological mechanisms in development. *Physiol. Revs.*, **34**, 1-24.

33. STEFFEE, C. H., WISSLER, R. W., HUMPHREYS, E. M., BENDITT, E. P., WOOLRIDGE, R. W., & CANNON, P. R. 1950. Studies in amino acid utilization. V. The determination of minimum daily essential amino acid requirements in protein-depleted adult male albino rats. *J. Nutrition*, **40**, 483-497.

34. STOKES, J. L., GUNNESS, M., DWYER, I. M., & CASWELL, M. C. 1945. Microbiological methods for the determination of amino acids. II. A uniform assay for the ten essential amino acids. *J. Biol. Chem.*, **160**, 35-49.

35. SZEPSENWOL, J., SHONTZ, M. E., & MASON, J. 1955. Nitrogen concentration in embryonic tissues of the chick throughout development. *Am. J. Physiol.*, **180**, 519-524.

36. WILKERSON, V. A., & GORTNER, R. A. 1932. The chemistry of embryonic growth. III. A biochemical study of the embryonic growth of the pig with specific reference to nitrogenous compounds. *Am. J. Physiol.*, **102**, 153-166.

37. WILLIAMS, H. H., CURTIN, L. V., ABRAHAM, J., LOOSLI, J. K., & MAYNARD, L. A. 1954. Estimation of growth requirements for amino acids by assay of the carcass. *J. Biol. Chem.*, **208**, 277-286.

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THE GROWTH-REPRODUCTION CYCLE

H. L. EICHHORN

(Received September 20, 1956)

We readily credit the high efficiency of the various functional units of our anatomy to evolution but the consideration of the pattern of the organism as a whole has seemed to be something to be avoided. Yet, just as surely as there are highly efficient patterns of the parts, there must have developed a highly efficient overall pattern of the entire organism.

Such a pattern was described in 1940 (2) and 1941 (3).

CLUE TO CYCLE

The discovery of the cycle came about not by project or assignment but quite by accident. In 1935, while making a study of the protein characteristics of two selected flocks of Leghorn hens during the period of increasing egg production, I was pleased to note (Fig. 1) the indication of a straight line relationship and a common slope ap-

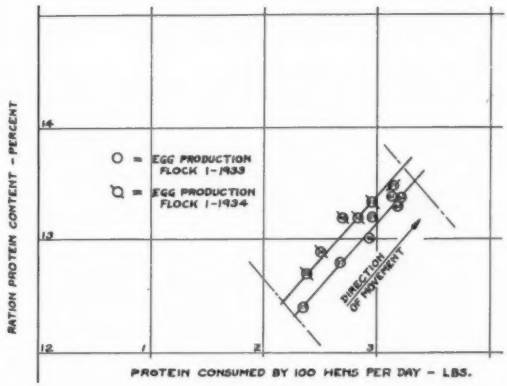

FIGURE 1

PROTEIN EXPANSION LINES—Protein characteristics of two selected flocks of Leghorn hens during period of increasing egg production.

plying to the two cases. When this slope was applied as a criterion in the feeding of other flocks, more characteristics appeared.

Effective feeding was found to lie between two well defined lines which I began to designate as limit lines. When a flock reached the upper "limit line", there appeared to be a tendency to follow this line to the left.

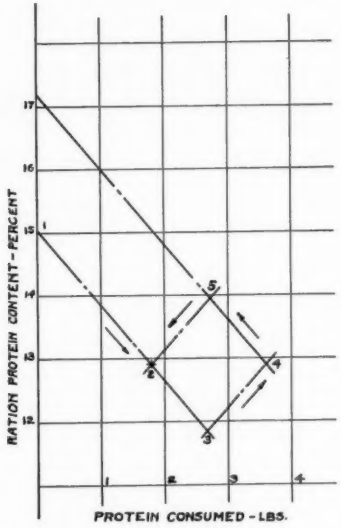

FIGURE 2
FIRST RATIONALIZATION OF CYCLE—Extension of Limit Lines of Figure 1 to protein content axis.

When the "limit lines" were projected to the protein content axis (Fig. 2), the points of intersection were found to be significant. The lower "limit line" intersected the axis near the point representing the protein content of egg yolk. Since the newly hatched chick is provided with a sac of egg yolk for first food, this line then became the approximation of the protein path of the growing pullet. The protein position of the flock at the close of the production period (Point 2) was known. This point was observed to lie very close to the protein path of growth, so that a closed cycle was indicated.

The upper "limit line" intersected the axis near the point representing the protein content of mash, the commercial feed mixture used as

a supplement to grain in feeding practice. This suggested that two supplementary components of the ration could be used for further study.

The transfer of the paths to ration component axes (Fig. 3), made solution possible and by re-application the protein paths of Fig. 2 eventually became Fig. 4.

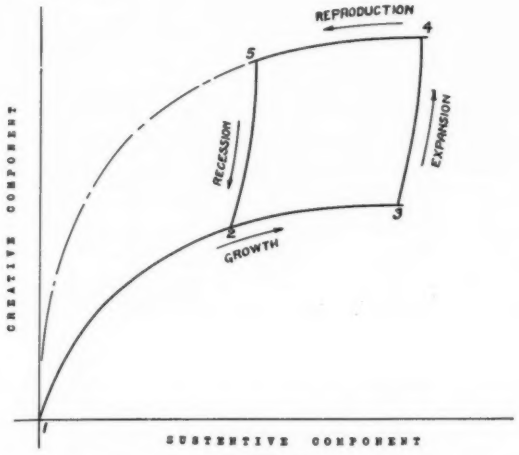

FIGURE 3

REPRODUCTION LOOP—Transfer of paths of Figure 2 to functional component axes.

Development and Verification

The object at this time is to simplify and clarify previous presentation and to add further evidence. To this end, the following statements seem justified:

(1) We and other living organisms are creatures of evolution. The instrument of evolution is selection. In countless incidents since life began, selection of structural and functional detail has been made on the basis of biological effectiveness or efficiency. The process represents a continuous improvement of pattern. It proceeds as an approach to a mathematical limit—the limit being the pattern of highest efficiency. There are therefore actually two life patterns: That which we observe today, the current pattern and that toward which this pattern is evolving, the ultimate pattern. With the passage of

time, the margin between the patterns has, in some cases, become quite small so that, when we speak of observing "perfection" of organ or process, we are actually pointing out portions of the ultimate pattern perceivable through the current pattern.

(2) The driving principle of life is continuity. In order to have continuity, there is reproduction. Reproduction requires a certain prepara-

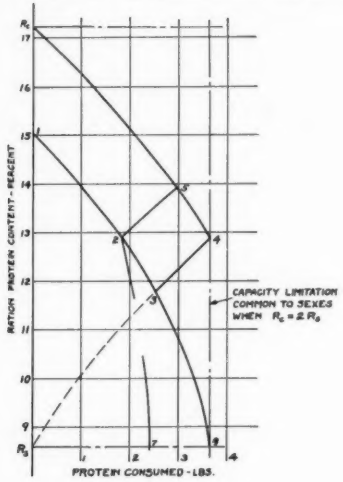

FIGURE 4
PROTEIN PATHS—Reconstruction of Figure 2 based on solution of most efficient pattern.

tion which we call growth. Growth, then, is not a primary activity. Its nature is determined by the requirements of reproduction on a level of highest biological efficiency.

(3) Total growth must occur in two stages. It would not be feasible for the parent organism to release offspring without mass. Such offspring would have no character and would therefore not represent reproduction in kind. Growth of offspring must be carried to a certain point by the parent. It is required that this growth, like all biological processes, be efficient. Since high efficiency would not be attained in conflict, the young organism must, for a certain period, be dependent upon and subservient to the parent. This period, which we will call minor growth, continues until the offspring is biologically ready

for further development as an accountable individual. There is then a change in course and major growth begins. The point where this change occurs we will call the point of transition. Point of transition is not to be confused with birth. Dependence may lie in a parental choice of environment, an organized supply of food or a period of special care essential to the proper development of the young of the species.

(4) A living organism converts food into body structure by growth and by reproduction. Food consumed in a given period of time by an efficient organism may be divided into two functional components as follows: The sustentive component furnishes the necessary materials for maintaining existing body structure and supplies the necessary energy for continued existence at a fixed level *without change in body mass*. The creative component is that portion which is required in addition to the sustentive component to increase body mass by growth or by reproduction. It consists of all materials which are necessary for increase in body mass but which would not be required if only existence at a fixed level of life and body mass were maintained.

* * * * *

The ultimate pattern, being the pattern of highest efficiency, is unquestionably subject to mathematical analysis and a beginning is readily made by portraying the most efficient application of the functional components.

In Fig. 5, point 0 represents the beginning of life of an organism—a mathematical representation with the mass of the organism at zero. With no mass to maintain, there is immediately no requirement for sustentive component and the first element of the growth path is therefore vertical. As growth proceeds, an increasing share of the ration (sustentive component) must be apportioned to maintain the increasing body mass leaving less of the ration proportionately available for further body mass increase (creative component). With the continued decrease in creative component increment, the path takes the convex form 0-3-9.

Minor growth appears at 0-1 and major growth at 1-9 with point 1, the point of transition. Creative component reaches a maximum at 3: then becomes zero at 9 and growth comes to an end.

Path 1-3-9 represents major growth without reproduction designation. In other words, it represents the growth path of the male. Where re-

production has been designated (female), creative component must be increased for that purpose and, under conditions of highest efficiency, will increase until the full capacity of the organism has been reached. Such a path, the path of expansion, is shown at 3-4.

Reproductive capacity begins at 3 and increases to a maximum at 4 while growth capacity decreases from a maximum at 3 to zero at 4. The full capacity of the female organism is reached at 4. From this point there can be only decrease. As the end of reproductive ac-

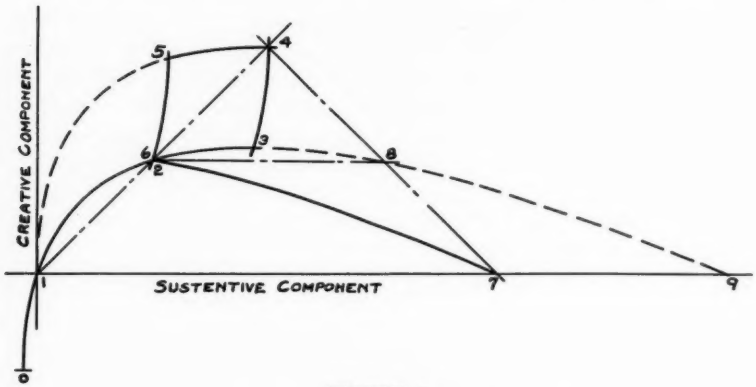

FIGURE 5
CYCLE OF SINGLE LOOP ORGANISM

tivity appproaches, so (under conditions of highest efficiency) must also the exhaustion of the organism approach. In other words, both functional components must now approach zero. This directs the course back toward the intersection of the component axes as along the path 4-5-1 (the reproduction path).

The figure 1-3-4-1 represents a simple reproduction cycle. It indicates the course followed by an organism which has but one period of reproduction with life ending when that period comes to an end. Our small grains would be found in this class. They grow along 1-3-4, moving to the level of full reproduction at 3-4. Along 4-1, reproduction continues at full capacity with that capacity on the decrease. As the sustentive component decreases along 4-1, maintenance comes to an end and, at 1, we find the familiar dead stalk of straw and chaff. When the seed is planted, germination begins at 0, minor (cotyledon)

growth extends to 1 and the cycle is repeated for the next generation.

In those cases in which life is not to end with the close of a single period of reproduction, another path comes into use. Such a path is shown at 5-6. Growth then reaches a maximum at 3 and decreases to zero at 4. Reproduction begins at 3, increases to a maximum at 4 and continues to 5. It then decreases to zero at 6. We will call 5-6 the path of recession. The figure 2-3-4-5-6 is a reproduction loop. The first contact of the growth path with the reproduction loop at

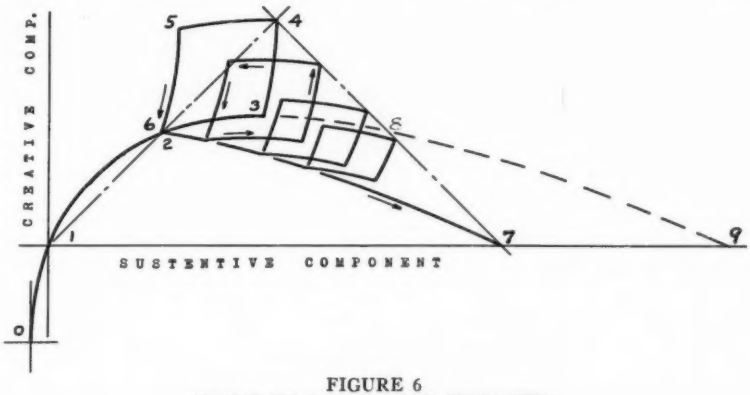

FIGURE 6
CYCLE OF MULTI-LOOP ORGANISM

point 2 is puberty. The contact of the recession path with the growth path (point 6) we will call loop closure.

It is to be noticed that travel along 4-5-6 is accompanied by a decrease in food capacity i.e. a decrease in capacity for function. This decrease is the cost of biological work done for the succeeding generation. The loss of capacity is regained later along the path of capacity recovery, 6-7. When additional reproduction loops are to be added (multi-loop organism, Fig. 6), these will lie between the path of capacity recovery and the line of skeletal limitation, 4-7. In such a case, the path of capacity recovery is followed segment by segment, the segments connecting succeeding reproduction loops.

Point 4 is the peak of efficient food consumption. Growth has come to an end; therefore the sustentive component is at its maximum. Reproductive capacity is at its maximum; so creative component is

at its maximum. The elements of the expansion and reproduction paths at point 4 are therefore vertical and horizontal respectively. Also since the pattern is one of highest efficiency, the cycle (as demonstrated in thermodynamics) is reversible, the paths are symmetrically placed with regard to the axes and the components at point 4 are equal.

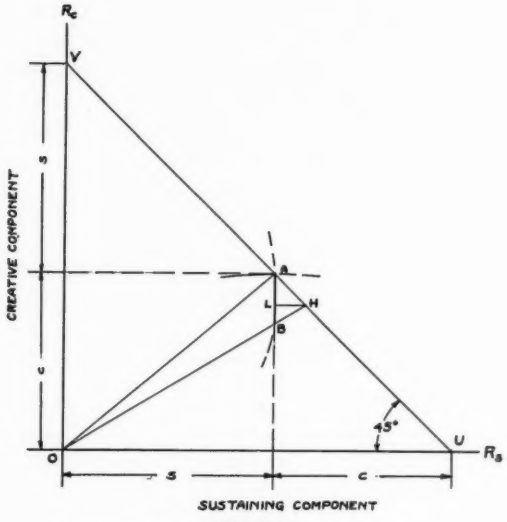

FIGURE 7
DERIVATION OF REPRODUCTION PATH

In Fig. 7, A with co-ordinates c and s represents the intersection of the expansion and reproduction paths at point 4. VU is the line of limiting food capacity 4f, through A. AB is the element of the expansion path.

$$AB = AL + LB = \left(\frac{AH}{VU}\right)(c + s) + \left(\frac{AH}{VU}\right)\left(\frac{c}{s}\right)(c + s)$$

$$= \frac{AH}{VU}\,\frac{(c + s)^2}{s}$$

$$s = \frac{AH}{(VU)\ (AB)}\,(c + s)^2$$

When $(c + s) = 4f$ $c = s = 2f$ and $\dfrac{AH}{(VU)(AB)} = \dfrac{1}{8f}$

$$c = \frac{(c + s)^2}{8f} \qquad (1)$$

$$s = \frac{(c + s)^2}{8f} \qquad (2)$$

These are expressions for the expansion and reproduction paths respectively.

* * * * *

In Fig. 8, OA represents the reproduction path; OB, the growth path of an organism and KL, a line of constant $(c + s)$ value.

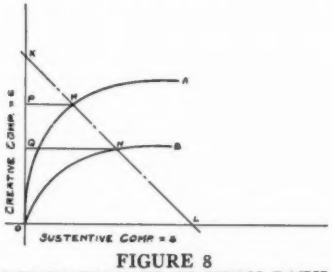

FIGURE 8
DERIVATION OF GROWTH PATH

At point M, $s = \dfrac{(c+s)^2}{8f}$ (Exp. 2)

At point N, $s = \dfrac{(c+s)^2}{8f} + QN - PM$

$OL = (c + s)$

$$s = \frac{(c+s)^2}{8f} + \frac{QN - PM}{OL}(c+s) = \frac{(c+s)^2}{8f} + R\,(c+s) \qquad (3)$$

This is a preliminary expression for the growth path.
Similarly, for the recession path,

$$c = \frac{(c+s)^2}{8f} + R\,(c+s) \qquad (4)$$

Solving for the food capacities or $(c + s)$ at the path intersections, we have at Col. 1

	Col. 1	Col. 2	
$(c+s)_1$	$= 0$	$= 0$	(5)
$(c+s)_2$	$= 4f(1 - 2R)$	$= 2f$	(6)
$(c+s)_3$	$= 4f(1 - R)$	$= 3f$	(7)
$(c+s)_4$	$= 4f$	$= 4f$	(8)
$(c+s)_5$	$= 4f(1 - R)$	$= 3f$	(9)
$(c+s)_6$	$= 4f(1 - 2R)$	$= 2f$	(10)
$(c+s)_7$	$= 4f$	$= 4f$	(11)
$(c+s)_9$	$= 8f(1 - R)$	$= 6f$	(12)

Points 1 and 9, Fig. 5 are points of placidity i.e. they are points without growth or reproductive activity. Point 6 is also a point of placidity since reproduction ends here (in the ultimate pattern) and growth is zero, having ended previously at point 4.

Now we note that the displacement between points 1 and 3 is $4f(1 - R)$ and the displacement between points 3 and 9 is also $4f(1 - R)$. This suggests that point 3 is a centroid of placidity and that the total path displacement from point 3 through points 4 and 5 to point 6 should also be equal to $4f(1 - R)$, or

$$(c + s)_4 - (c + s)_3 + (c + s)_4 - (c + s)_6 = 4f(1 - R)$$

Applying values of Col. 1, $R = \frac{1}{4}$

This provides the food capacity values at the path intersections as given in Col. 2.

The expressions for the paths now become

Growth $$s = \frac{1}{8f}(c + s)^2 + \frac{1}{4}(c + s) \qquad (13)$$

Expansion $$c = \frac{1}{8f}(c + s)^2 \qquad (14)$$

Reproduction $$s = \frac{1}{8f}(c + s)^2 \qquad (15)$$

Recession $$c = \frac{1}{8f}(c + s)^2 + \frac{1}{4}(c + s) \qquad (16)$$

* * * * *

Since c and s are maximum at point 4, this point corresponds to the maximum of the (c + s)-time curve. 2-3-4-5-6, being a cycle of highest efficiency, is reversible. (Actual reversal of paths of the growth-reproduction cycle is not uncommon.) The (c + s)-time curve is therefore symmetrical about a vertical axis. Examination of the feed consumption of animals indicates that the feed consumption-time relationship is parabolic. (See Fig. 9A). This seems logical since the cycle paths (in terms of c and s) have been found to be parabolic (Exp. 13 to 16). The expression for such path is

$$(c + s) = \frac{4f}{h}\left(2t - \frac{t^2}{h}\right) \qquad (17)$$

in which t is elapsed time and h is time required to reach maximum food capacity.

* * * * *

The expression for the reproduction path was found on the basis of pattern of highest efficiency. The expression for the growth path was developed from the reproduction path on the same basis. Creative component units consumed during growth measure the increase of mass during the period of growth. Therefore we can arrive at the nature of efficient growth by the summation of creative component units during the period of growth.

The expression for the path of major growth was found to be

$$s = \frac{1}{8f}(c + s)^2 + \tfrac{1}{4}(c + s) \qquad \text{(Exp. 13)}$$

Creative component applied along the growth path is

$$c = (c + s) - s = \tfrac{3}{4}(c + s) - \frac{1}{8f}(c + s)^2$$

The amount of creative component applied to increase body mass in any period of time is

$$\int_{t=0}^{t=\phi(c+s)} c\, d(c + s)$$

The actual body mass resulting from such application of creative component is some portion of this. Let p represent this portion. Also

let w represent body weight at any time and W_h, the body weight at end of growing period.

$$w = p \int c\, d(c+s) = p \int \left[\tfrac{3}{4}(c+s) - \frac{1}{8f}(c+s)^2 \right] d(c+s)$$

$$= p \left[\tfrac{3}{8}(c+s)^2 - \frac{(c+s)^3}{24f} \right]$$

In the case of the female, $w = W_h$ when $(c+s) = 4f$ (Exp. 8)

$$w = \frac{3W_h}{10f^2} \left[\frac{3(c+s)^2}{8} - \frac{(c+s)^3}{24f} \right]$$

Applying Exp. 17,

$$w = \frac{W_h}{5h^2} \left[9 \left(2t - \frac{t^2}{h} \right)^2 - \frac{4}{h} \left(2t - \frac{t^2}{h} \right)^3 \right] \qquad (18)$$

or $$w = \frac{W_h}{5_h{}^2} \left[9z^2 - \frac{4z^3}{h} \right] \text{ when } z = \left(2t - \frac{t^2}{h} \right) \qquad (18A)$$

In the case of the male $w = W_h$ when $(c+s) = 6f$ (Exp. 12)

$$w = \frac{W_h}{h^2} \left[3z^2 - \frac{2z^3}{h} \right] \qquad (19)$$

In Fig. 9, Exp. 17 and 18 are compared with data taken on Leghorn hens. The close registration indicates that the Leghorn hen has already approached quite close to the ultimate pattern. This may be attributed to the critical selection applied in the evolution of a flying creature and to the subsequent selection by man of individuals having the highest reproductive efficiency.

Man, as seen in Fig. 10 and 11, is still biologically young. The sigmoids of minor and major growth are not completely developed but the transition dip is quite in evidence and one may conclude that the characteristics and effects of transition are not to be ignored.

* * * * *

In the case of the single reproduction loop with capacity recovery path, the human cycle—Fig. 5, the times at which the various path

junctures occur may be found by applying Exp. 17 to Exp. 5 to 11 with results as follows:

$$t_1 = 0 \qquad (20) \qquad t_5 = 1\tfrac{1}{2}h \qquad (24)$$
$$t_2 = (1 - \sqrt{\tfrac{1}{2}})h \qquad (21) \qquad t_6 = (1 + \sqrt{\tfrac{1}{2}})h \qquad (25)$$
$$t_3 = \tfrac{1}{2}h \qquad (22) \qquad t_7 = (1 + 2\sqrt{\tfrac{1}{2}})h \qquad (26)$$
$$t_4 = h \qquad (23)$$

The ages at which the various path junctures occur may be found when the ages at any two junctures are known. Using the human

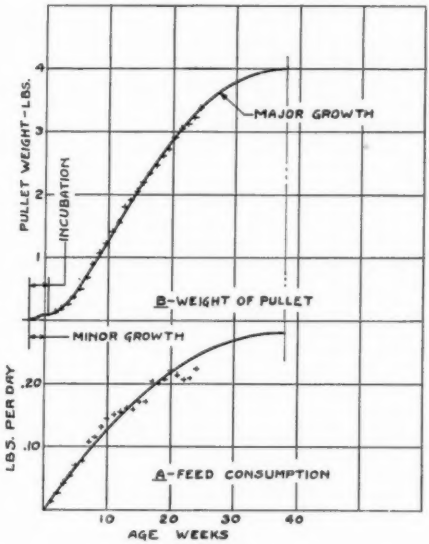

FIGURE 9
FEED CONSUMPTION & GROWTH—LEGHORN HEN
STORRS BUL. 96

female as an example (3, p. 357), if point 2 is taken to occur at the age of 12 and point 7 is taken to occur at the age of 50, then

$$(1 + 2\sqrt{\tfrac{1}{2}})h - (1 - \sqrt{\tfrac{1}{2}})h = 50 - 12$$
$$h = 17.9$$

Since puberty takes place $(1 - \sqrt{\tfrac{1}{2}})h$ or 5.25 years after transi-

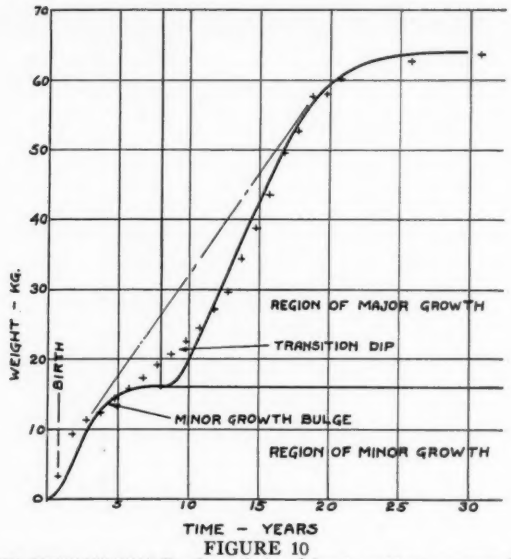

FIGURE 10

GROWTH OF HUMAN MALE—Growth in ultimate pattern compared with data by Quetelet.

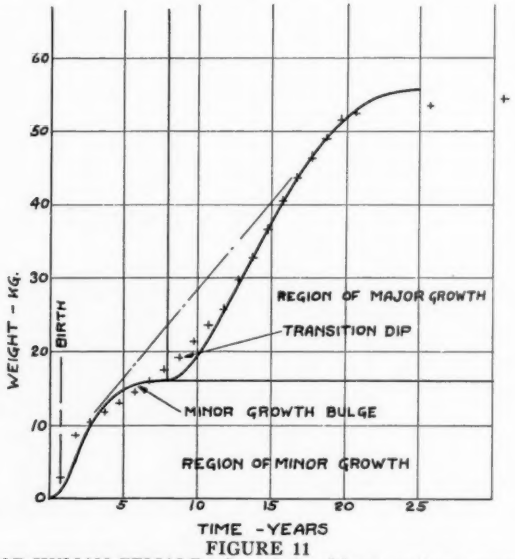

FIGURE 11

GROWTH OF HUMAN FEMALE—Growth in ultimate pattern compared with data by Quetelet.

tion, transition occurs at the age of 12 — 5.25 or 6.75 years. Proceeding further, other junctures are found to occur at the following ages:

3	Optimum Inception	15.7
4	Maturity	24.6
5	Recession Begins	33.6
6	Loop Closure	37.3
7	Recovery Ends	50.0

This age pattern may vary somewhat according to ethnical group or to the individual. We will refer to it as the 6.75-17.9 pattern.

* * * * *

According to the ultimate pattern, reproductive capacity becomes zero at loop closure. It is common knowledge that human reproduc-

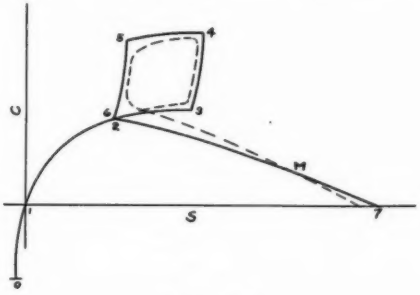

FIGURE 12
COMPARISON OF ULTIMATE AND CURRENT PATHS (hypothetical).

tion (in the current pattern) may continue after the age of 37. This situation (evidence of the incompleteness of our evolution) may be explained with the help of Fig. 12.

In Fig. 12, the course followed by an individual or group in the current pattern (shown dotted) is necessarily hypothetical since research in this field seems to be non-existent. While the paths intersect sharply in the ultimate pattern, we would expect the intersections to be rounded (less perfect) in the current pattern. As long as the course lies above the path 6-7, reproduction may occur. The possibility continues until contact is made with the capacity recovery path at M and M (menopause) may occur therefore at any time between the vicinity of point 6 and point 7.

Reproduction may occur after the time of loop closure but as the actual path closely approaches or crosses the capacity recovery path of the ultimate pattern, it seems logical to expect cases of some specific "irregularity". Might mongolism "after forty" be that "irregularity"?

* * * * *

An acceptable growth expression should certainly be sigmoid and be in terms of the single variable, time. But this is not all. The segments of increasing and decreasing increments should be in correct proportion to each other. In other words, the point of inflection should be correctly located.

It has been found (5, p. 31) that the inflection point of the growth curve of an animal is concurrent with puberty. Puberty occurs at $t = (1 - \sqrt{1/2})h$ (Exp. 21) and the point of inflection may be accurately determined by second derivative.

The second derivative of Exp. 18 is

$$\frac{d^2w}{dt^2} = \frac{W_h}{5h^2}\left[\, 72 - \frac{408t}{h} + \frac{684t^2}{h^2} - \frac{480t^3}{h^3} + \frac{120t^4}{h^4} \,\right] \qquad (27)$$

When $t = (1 - \sqrt{1/2})h$, $\dfrac{d^2w}{dt^2} = 0$

The point of inflection of Exp. 18 does therefore occur at puberty, agreeing with recorded observations.

It is very doubtful that any growth expression which has not been based on the most efficient pattern of life would pass such a test.

* * * * *

In the 6.75-17.9 pattern for the human female submitted as an example in 1941 (3, p. 357), loop closure occurs at the age of 37.3.

In 1951 (1), Dr. Gunnar Dahlberg published his curves on the occurrence of twins and triplets with respect to age of mother. The curves were quite similar—each reaching a sharp peak near the age of 37.5 (see Fig. 13).

In the light of the growth-reproduction cycle, Dr. Dahlberg's peaks may be explained as follows: Animals, which are normally limited to one offspring per birth, have a mechanism which functions accordingly. In an organism of highest efficiency (following the solid lines of Fig. 12), the functioning of such a valving mechanism would de-

crease as the need for function decreased, becoming zero at loop closure.

The "valving" mechanism has been in operation not only in single loop man but previously in his multi-loop ancestors. In all this time, this mechanism has been approaching perfection—perfection dictating functional decrease to zero at loop closure.

On the other hand, because of the incompleteness of the evolution of man (as indicated by the dotted lines of Fig. 12) there is a tendency in the current pattern for reproductive capacity to over-run loop closure, declining to zero at M.

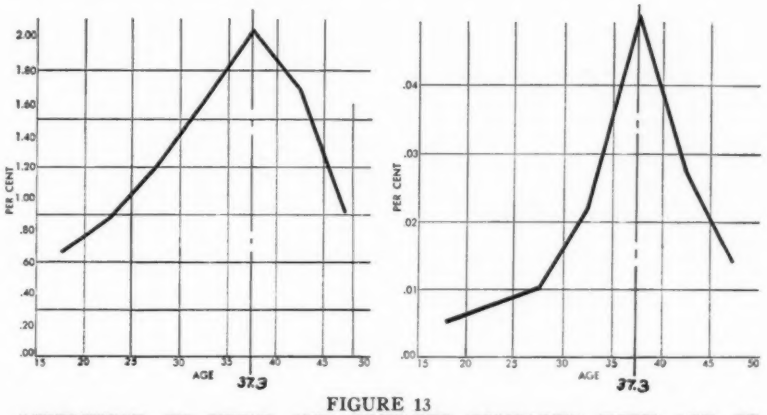

FIGURE 13
LIKELIHOOD OF TWINS AND TRIPLETS COMPARED WITH AGE OF MOTHER—Peak agrees closely with age at loop closure.

So one would expect gathered data to show an increase in the likelihood of multiple births followed by a decrease, with the point of maximum at loop closure. Thus loop closure is indicated at the age of 37.5 through the work done by Dr. Dahlberg and at the age of 37.3 by the growth-reproduction cycle pattern described in 1941.

The peak discovered by Dr. Dahlberg definitely represents a biological change; the change being that which takes place at loop closure. The publication of the biological changes of the human female in 1941 was, in essence, a prediction. The prediction dealt not only with the fact of the existence of a biological change but with the time of occurrence of that change.

* * * * *

It is felt that this is little more than an introduction to an extensive field of research very essential to the full understanding of life and to the physical and psychological welfare of man himself. This field is so individualistic as to deserve a category of its own and,

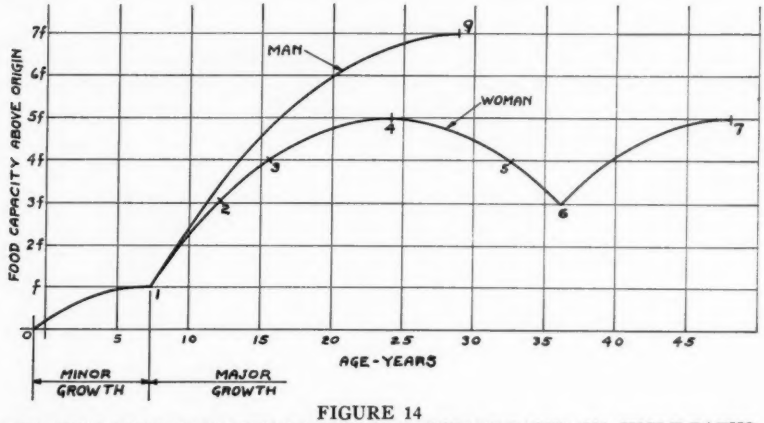

FIGURE 14
RELATIVE FOOD CAPACITY OF MAN AND WOMAN BASED ON CYCLE PATHS.

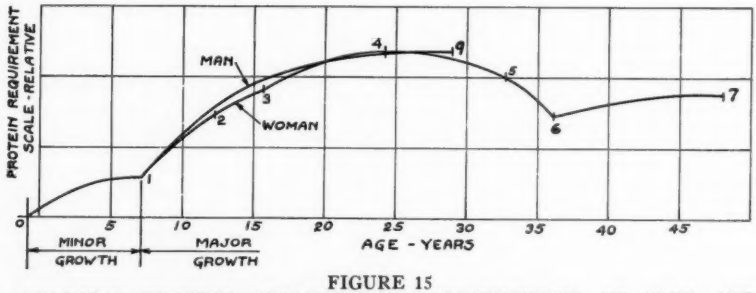

FIGURE 15
RELATIVE PROTEIN QUANTITATIVE REQUIREMENT OF MAN AND WOMAN BASED ON CYCLE PATHS.

taking a cue from thermodynamics, I suggest that it be named vitodynamics. It holds prospect of fulfilling all that the term implies.

To some extent, man is now directing his own evolution and it is essential that this be done with the fullest understanding. Indeed, those maladies commonly called "diseases of civilization" may only represent cases where we are out of step with what we are.

My own ignorance should forbid further discussion but being still quite alone, must speak for myself. I would call attention to the stress which seems to exist at transition as indicated at 1 in Fig. 14 and 15. To this may sometimes be added the stress of premature school pressure. It is suggested that the child be given more privilege in setting his own pace at this time. Better minds and healthier nervous systems might result. Such a warning comes also from another quarter (4, p. 708). One finds here that competent investigation has shown that the start of serious academic training might better be

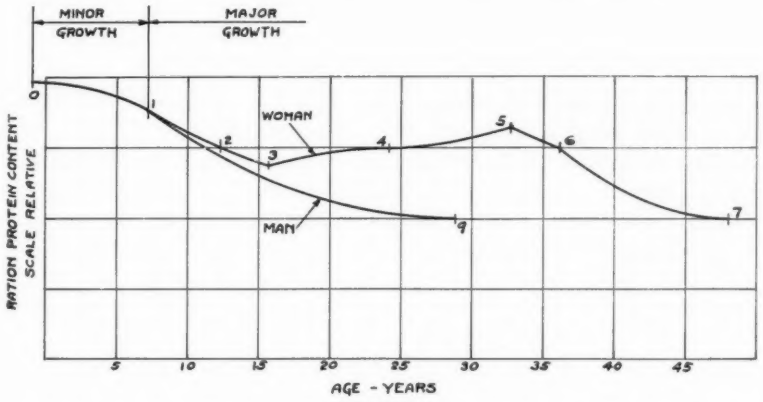

FIGURE 16
RELATIVE PROTEIN CONTENT REQUIREMENT OF MAN AND WOMAN BASED ON CYCLE PATHS.

transferred from the age of 6 to the age of 7 or 8 thus relieving instead of compounding the period of stress which the cycle indicates. It is essential that more be known about transition. The critical nature of the change may be a factor in the high incidence of "childhood diseases" at this time. The midget appears to be an individual who has somehow been blocked at transition and has then failed to make the complete shift from minor growth to major growth.

I would call attention to the indication of increased protein requirement for women between the ages of 16 and 33 as shown in Fig. 16. Compare with the curve for men. Evidently women should be the "meat eaters" during these years. Many of them would be thankful if more attention to the protein content of their diet had saved them

the discomfort and unsightliness of varicose veins as a by-product of childbirth. Other and more serious maladies must certainly result from failure to fill indicated protein requirements.

On Fig. 14, 6-7 indicates what takes place along the path of capacity recovery ("the fat forties"). It has not been possible, so far, to plot the path of men after the period of growth (point 9). Their changes come largely through biological association. Is it possible that the systems of men are sometimes overly influenced by the path of capacity recovery as a human heritage and must then inconveniently (sometimes fatally) store materials which, in the case of women, would represent replenishment after reproduction?

* * * * *

These are crude indications of the analytical value of the cycle. What of its value as a differential of the species?

What paths are used by insects and microscopic life? Might understanding of the cycle paths suggest more effective pest control? How does the bee control cycle paths and manipulate the requirement for functional component?

On the quantitative side: the cycle indicates that a "cost" is involved in reproduction. How can this be measured and definitely expressed? Is there any doubt that vital energy and vital work will some day be expressed in definite terms? What limit can there be to research in this field?

Conclusion

The writer feels that he should ask the indulgence of the reader for he is, as may easily be surmised, not a biologist but an engineer.

Observing evidence of the operation of a closed cycle in the life of an animal and feeling that any such operation could scarcely have gone unobserved by others, he set about to find his error. Each test made, however, seemed to prove that no error was involved, that the cycle did actually exist and that it had general application. In the intervening years, other tests have been applied with the same result.

Two such tests seem to deserve repetition. (1) The growth expression developed by strict mathematical methods from the cycle paths is a complete sigmoid and the point of inflection is located precisely at puberty—agreeing with recorded observations. (2) The pattern of human biological changes published in 1941 predicted a

change at the age of 37.3. In 1951, Dr. Dahlberg found by an entirely independent method a biological change at 37.5.

If the growth-reproduction cycle does exist, it is very essential to human welfare that it be understood to the fullest extent. The study should reveal a new field of research which promises to be quite extensive and a plea is made that the possibilities be investigated.

References

1. Dahlberg, Gunnar. An Explanation of Twins. *Sci. Am.* **184**, 47-51.
2. Eichhorn, H. L. The Growth Reproduction Cycle. Growth, **4**, 1-16.
3. ————. The Growth Reproduction Cycle. Growth, **5**, 351-358.
4. Scott, G. D. Nutrition of Infants and Children. Chapman and Grimes, Boston. Pp. 778.
5. Univ. of Missouri Res. Bul. 97.

Growth, 1956, **20**, 265-273.

EFFECT OF A GLUCOSE ANALOG ON NUCLEIC ACIDS IN TUMOR TISSUE*

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In a previous paper (17), we reported that a vitamin C-free diet resulted in a decrease in the total and nuclear DNA contents in the fibrosarcoma AL-2 of guinea pigs, and that a similar effect, although of a lesser degree, was obtained upon administration of the ascorbic acid analog, D-gluco-ascorbic acid to rats bearing the Crocker rat carcinoma. The findings of Horowitz and King (8), that glucose is a precursor of ascorbic acid, with a synthesis proceeding by way of D-glucuronolactone, suggested the desirability of investigating the effect of glucose analogs on tumor growth. The work of Bernstein (2), Akabori *et al.* (1), Horecker (7), Low (10), Plaut (12), and Schmitz *et al.* (15) indicated that the pentoses of PNA and DNA are probably formed from glucose metabolites by the action of enzymes, although the exact pathways by which pentose formation proceeds are not known.

In the present study, 2-deoxy-D-glucose (2-DG), an analog of glucose which differs from it by the absence of one oxygen atom at the second carbon atom, was investigated. Cramer and Woodward (5) have shown that 2-DG competes with glucose in the yeast fermentation system and inhibits fermentation of glucose. Woodward and Hudson (20) also reported that the analog inhibits the anaerobic glycolysis of rat tumor slices; an inhibition of 72-91 per cent was observed in both Flexner-Jobling and Walker 256 carcinoma. Tumor glycolysis was inhibited aerobically as well as anaerobically. 2-DG does not significantly affect the endogenous respiration of the tissues,

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normal or malignant, at least in those concentrations required to inhibit glycolysis. The type of inhibition appears to be competitive, since inhibition of glycolysis was prevented by increasing the concentration of glucose in the medium surrounding the tumor slices. Wick *et al.* (19) showed that 2-DG rapidly enters the cells of the extrahepatic tissues in the absence of insulin, and that insulin administration appears to increase the rate of cell entry. Thus, the glucose analog, by inhibiting the transfer and oxidation rates of glucose acts as a metabolic block for glucose. The investigators suggested that the blocking of glucose may take place at the 6-phosphate stage. Sokoloff *et al.* (18) reported that a diet supplemented with 2-DG inhibited the growth of transplanted Crocker rat carcinoma. Ely (6) reported that the subcutaneous administration of 2-DG increased the survival time of mice bearing Krebs ascites carcinoma, but had no effect on the survival of C57 black mice inoculated with myelogenous leukemia C1498.

Materials and Methods

Rat carcinoma G-175, originally obtained from the Crocker Cancer Laboratory, Columbia University and mouse sarcoma 180 were used in this study. The anti-tumor activity of 2-DG was investigated both by oral administration and subcutaneous injections of the compound to rats, bearers of carcinoma. A standard seven-day test was used on mice bearers of sarcoma 180. Both species of animals were placed on an exclusive meat diet, consisting of fat free chopped beef meat, for three days prior to transplantation and for eight days afterward (standard test). The meat diet was well tolerated and did not affect the growth of tumors to any significant degree. The tumors used for the determination of nucleic acids were 1.0-3.0 cm. in diameter, fast-growing and without necrotic areas.

Two methods were used for determining the plasma and tissue concentration of 2-DG: one, the Miller-Van-Slyke method, based on the observation that 2-DG gives negligible reducing values for blood sugar when the heating time is eight minutes; two, the procedure of Cramer and Neville (4), based on a quinaldine reaction for alpha-methylene aldehydes.

A slightly modified method of Barnum *et al.* (3) and of Payne *et al.* (11) was used for the extraction and separation of DNA and PNA, as described by us in our previous publication. The technique

of Schneider (16) was used for the estimation of DNA and PNA in the tumor tissue.

The procedure for the estimation of the nuclear DNA was essentially the same as that recommended by Lessler (9). Tissues were fixed in 6 per cent neutral formalin, washed in running tap water, dehydrated, embedded in paraffin, sectioned, hydrated, and hydrolyzed for 12 minutes in 1 N HCl at 55° C. Following the hydrolysis, the preparations were rinsed in running water for 5 minutes, washed twice in distilled water, and exposed to the Feulgen reagent for 1 hour and 30 minutes, placed in 3 changes of water saturated with sulfur dioxide, and washed twice in distilled water. To remove the yellow dye impurity in the Feulgen reaction, sulfur dioxide was bubbled through a 0.5 per cent basic fuchsin solution for two hours, and 1 gm. of decolorizing charcoal was added to 200 cc. of solution; the solution was then agitated and filtered. The spectrophotometric apparatus and technic employed in this investigation were similar to those described by Pollister (13). The apparatus consisted of a photomultiplier tube mounted above a microscope. The DNA content per cell, in arbitrary units, was calculated according to the procedure of Lessler.

Results

Toxicity of 2-DG.—The analog, added to the ration of adult rats to the extent of either 2 or 5 per cent, was well tolerated by them. There was no significant loss in weight as compared with control animals. Thirty male rats, average weight 233 gm., were arranged in three groups of ten rats each: (a) controls, on iso-caloric Purina ration; (b) the Purina ration supplemented with 2 per cent of 2-DG; and (c) the ration supplemented with 5 per cent of the analog. The average weight after three weeks on the regimens described was 261 gm. for the first group; and 259 and 253 gm., respectively, for the two treated groups.

In contrast to the insensitivity of adult rats to 2-DG, the growth of young rats was significantly inhibited by the analog. The initial average weight of 30 rats was 83 gm. After three weeks, the control group of 10 animals average 137 gm., while the group of 10 rats on 2 per cent of 2-DG was 109 gm. (20 per cent inhibition) and the group on 5 per cent was 91 gm. (33 per cent inhibition). Adult mice and rats tolerated well daily subcutaneous injections of 500 mg. of

2-DG per kg. of body weight for 20-25 days. No loss of weight was observed. The analog was detected in blood plasma and the tissues of rats during the period from ½ to 8 hours after subcutaneous injection of a single dose of 100 mg.; it appeared in the urine in about 40-45 minutes after the injection. Only small amounts (5-6 μg/10 mg. tissue) of 2-DG were discovered in homogenates of rat carcinoma 1-4 hours following the subcutaneous injection of 100 mg. However, when 500 mg. of 2-DG was injected, much larger amounts of the analog, up to 15 μg/10 mg., were detected in tumorous tissue.

Anti-tumor activity of 2-DG.—Ninety rats of an average weight of 205 gm. were divided into three groups, each of 30 animals, and fed on a meat diet. Group I served as the control; to the diet of group II, 2 per cent of 2-DG was added; while that of group III was supplemented with 5 per cent of the analog. Carcinoma G-175 was transplanted into each of the animals and the tumor size was measured every five days. By the 24th day, the average size of the tumor in Group I was 15.2 $cm.^3$ in Group II—8.4 $cm.^3$; and in Group III—6.3 $cm.^3$ Chart I summarizes the results of these trials.

The average span of life of treated rats was higher as compared with the control group. The average survival time of the rats of group I was 25 days, that of the animals of group II was 32 days, and that of the rats of group III was 42 days. Chart II summarizes the results.

Thirty mice, bearers of sarcoma 180, were divided into 3 groups and used for the standard test with 2-DG. Group I served as control; the animals of Group II were given 2-DG, 50 mg. subcutaneously once daily, while those of Group III were given the same dose twice daily, for seven days. The treatment was started the day after transplantation. On the eighth day, the tumors were excised and weighed. Group II showed an average inhibition of 27 per cent and Group III of 33 per cent, as compared with the controls.

Fifteen rats, bearers of carcinoma G-175, average weight 245 gm., were divided into three groups of 5 animals each. Of these, Group I served as the control, while the animals of Group II were given subcutaneously 500 mg. and those of Group III, 1000 mg. of 2-DG daily for seven days, beginning 24 hours after transplantation of the tumor. Group II gave an inhibition index of 22%, and Group III of 28%.

The trials with rat carcinoma G-175 and mouse sarcoma 180 indi-

cate a moderate, but definite, anti-tumor activity of the glucose analog, 2-deoxy-D-glucose.

Total PNA and DNA content of rat carcinoma.—Thirty male rats, average weight 187 gm., were divided into three groups, ten animals each, and fed isocalorically on a meat ration. Group I, control; for Group II the ration was supplemented with 2 per cent of

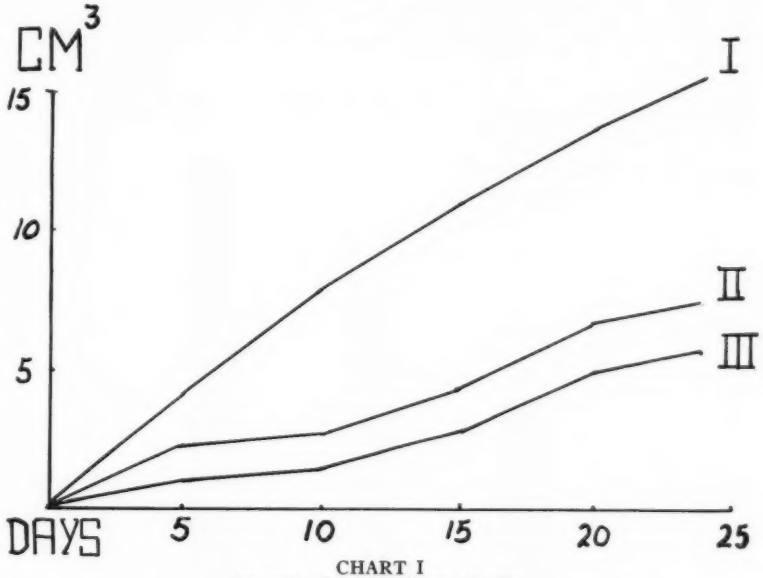

CHART I
Growth of rat carcinoma G-175.
Group I, control, 30 rats, isocaloric meat diet.
Group II, 30 rats, isocaloric meat diet supplemented with 2% of 2-DG.
Group III, 30 rats, isocaloric meat diet supplemented with 5% of 2-DG.

2-DG, and for Group III, with 5 per cent of the analog. Carcinoma G-175 was transplanted to all animals after 24 hours on these diets. The tumors were removed 8 days after transplantation when they were approximately 2.5 cm. in diameter. Twelve hours before excision of the tumors, food was withdrawn from the animals. The total PNA and DNA of tumor tissues was estimated following Schneider's technique (16). Table I summarizes the results of this study.

The data of Table I indicated that there was a decrease in the

DNA content of rat carcinoma G-175 when the animals were kept on a diet supplemented with the analog. This shift in the PNA/DNA ratio was larger with 5 per cent than with 2 per cent of 2-DG, amounting to 25 per cent.

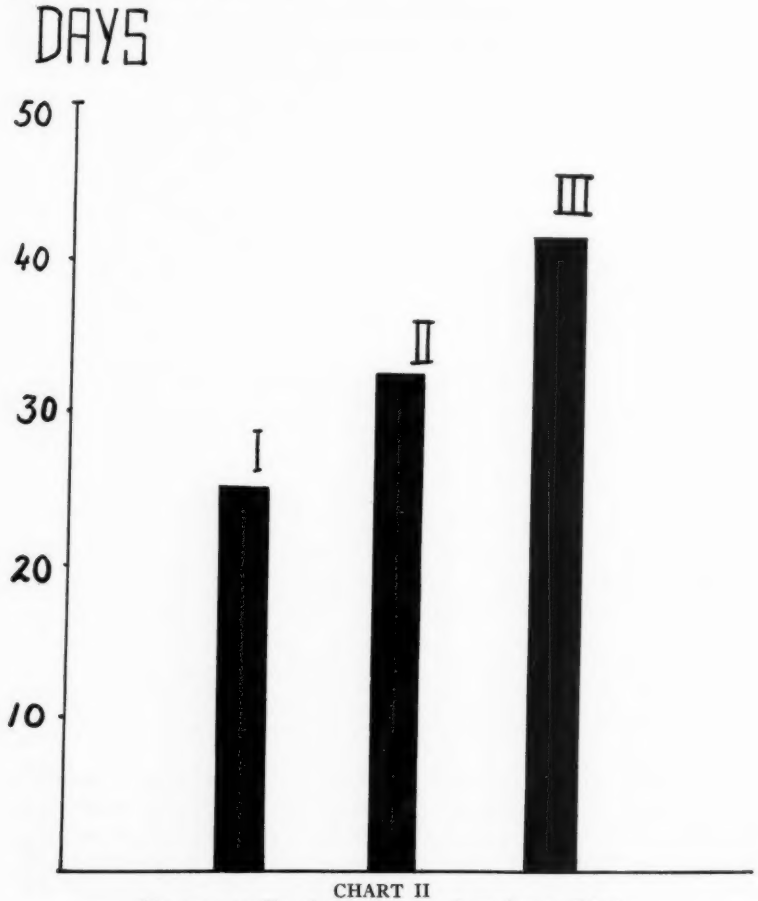

CHART II

The span of life of rats, bearers of carcinoma G-175.
Group I. Control group.
Group II. On 2 per cent of 2-DG.
Group III. On 5 per cent of 2-DG.
Each group of 30 rats on isocaloric meat diet.

Nuclear DNA content of rat carcinoma.—In another series of experiments, the nuclear DNA content of rat carcinoma G-175 was estimated with the Feulgen reaction. Thirty male rats, average weight of 165 gm., were divided into three groups and treated in a manner identical to that of the previous experiment. The tumors were removed 8 days after transplantation and were about 2.3 cm. in diameter. Table II gives the results of this estimation. The DNA nuclear content is expressed in arbitrary units calculated after the extinction coefficient of the nucleus was multiplied by the area.

TABLE I
Effect of 2-Deoxy-D-Glucose on the PNA and DNA Content in Rat Carcinoma G-175.

Group	Supplement	No. Tumors	PNA per cent of dry wt.	DNA per cent of dry wt.	PNA/DNA ratio
I	None	5	2.23 ± 0.13	2.97 ± 0.19	0.75
II	2 per cent 2-DG	6	2.15 ± 0.18	2.32 ± 0.11	0.81
III	5 per cent 2-DG	6	2.02 ± 0.10	2.04 ± 0.10	1.0

TABLE II
Effect of 2-Deoxy-D-Glucose on the Nuclear DNA Content in Rat Carcinoma G-175. Feulgen Reaction.

Group	Supplement	No. Specimens*	No. Cells	(E) x (A)** Arbitrary Units
I	None	4	75	3.95 ± .09
II	2 per cent 2-deoxy-d-glucose	5	100	3.35 ± .07
III	5 per cent 2-deoxy-d-glucose	6	125	3.02 ± .14

* Each specimen taken from a different tumor.
** E = extinction coefficient.
A = nuclear area, in D^2.

The figures of Table II suggest that the analog administered to the rats bearers of carcinoma G-175 in amounts of 2 and 5 per cent for a period of 8 days brings down the nuclear DNA content in the tumor tissue. This decrease amounts to about 10 per cent for 2 per cent, and to about 20 per cent for 5 per cent of the analog. There was also recorded a drop in the total DNA content of carcinoma G-175 resulting from administration of 2-DG.

Discussion

Potter and Heidelberger (14) postulated that "growth in a special tissue represents a response to a need, while self-limitation of growth is brought about by the removal of certain essential building blocks," and that a clear distinction should be made between essential metabolites and essential nutrients when one refers to the essential building blocks of growth. Both the ribose and deoxyribose fractions of the nucleic acids should be considered as essential metabolites in the growth of animal tissues. Schmitz *et al.* (15) demonstrated in their studies on alternative pathways of glucose metabolism in tumor tissues *in vivo* that both the PNA and DNA of the tumors incorporated considerable amounts of radio-active glucose after a single intraperitoneal injection of glucose-1-C.[14] This work indicates the important role which glucose metabolites play in the formation of the PNA and DNA of tumor tissue. Our present studies with 2-deoxy-D-glucose have shown that this analog of glucose interferes with the formation of the PNA and DNA in tumor tissues, and inhibits the growth of tumors. The work of Potter and Schmitz *et al.*, substantiated by the present investigation, emphasizes the necessity of further search for agents capable of interfering with some of the functions of glucose.

Summary

2-Deoxy-D-glucose, an analog of glucose, inhibited the growth of rat carcinoma G-175, when added to the ration at levels of either 2 or 5 per cent. Although adult rats tolerated this diet well, the growth of young rats was retarded by administration of the analog at these levels.

2-Deoxy-D-glucose inhibited the growth of mouse sarcoma 180 and rat carcinoma G-175 when injected subcutaneously daily for seven days at a level of either 2.0 or 4.0 gm./kg. per day.

2-Deoxy-D-glucose decreased the total and nuclear DNA content in rat carcinoma G-175.

References

1. Akaeori, S., Uehara, K., & Muramatzu, I. 1952. On the Biochemical Formation of Monosaccharides. *Proc. Japan Acad.*, **28**, 39-43.
2. Bernstein, I. A. 1953. Synthesis of Ribose by the Chick. *J. Biol. Chem.*, **205**, 317-31.
3. Barnum, C. P., Nash, C. W., Jennings, E., Nygaard, O., & Vermund, H. 1950. The Separation of Pentose and Desoxypentose Nucleic Acids from Isolated Mouse Liver Cells Nuclei. *Arch. Biochem.*, **25**, 376-83.

4. CRAMER, F. B., & NEVILLE, G. A. 1953. A Colorimetric Method for Estimating 2-Desoxyglucose. *J. Franklin Inst.*, **256**, 379.

5. CRAMER, F. B., & WOODWARD, G. E. 1952. 2-Deoxy-D-glucose as an Antagonist of Glucose in Yeast Fermentation. *J. Franklin Inst.*, **253**, 354-60.

6. ELY, J. O. 1954. 2-Deoxy-D-glucose as an Inhibitor of Cancerous Growth in Animals. *J. Franklin Inst.*, **258**, 157-60.

7. HORECKER, B. L. 1951. The Metabolism of Pentose and Triose Phosphates. In W. D. McElboy and B. Glass (eds.), Phosphorus Metabolism, **1**, 117-45. Baltimore: Johns Hopkins Press.

8. HOROWITZ, H. H., & KING, C. G. 1953. Glucuronic Acid as a Precursor of Ascorbic Acid in the Albino Rat. *J. Biol. Chem.*, **205**, 815-21.

9. LESSLER, M. A. 1953. The Nature and Specificity of the Feulgen Nuclear Reaction. *Internat. Review of Cytology*, **2**, 231. New York: Academic Press.

10. LOW, B. 1952. Mechanism of Ribose Synthesis in the Rat. *Acta Chem. Scandinav.*, **6**, 304.

11. PAYNE, A. H., KELLY, L. S., & WHITE, M. R. 1952. Effect of Neoplastic Tissue on the Turnover of Liver Nucleic Acids. *Cancer Research*, **12**, 65-68.

12. PLAUT, G. W. E. 1953. Mechanism of Riboflavin Synthesis. *Fed. Proc.*, **12**, 254-55.

13. POLLISTER, A. W. 1952. Photomultiplier Apparatus for Microspectrophotometry of Cells. *Lab. Investigation*, **1**, 106.

14. POTTER, V. R. & HEIDELBERGER, C. 1950. Alternative Metabolic Pathways. *Physiol. Rev.*, 30, 487-512.

15. SCHMITZ, H., POTTER, V. R., & HURLBERT, R. B. 1954. Alternative Pathways of Glucose Metabolism. I. Distribution of Radioactivity from Glucose-1-C^{14} in Acid-soluble and Acid-insoluble Fractions of Tumor and Normal Tissue. *Cancer Research*, **14**, 58-64.

16. SCHNEIDER, W. C. 1945. Phosphorus Compounds in Animal Tissues. I. Extraction and Estimation of Desoxypentose Nucleic Acid and of Pentose Nucleic Acid. *J. Biol. Chem.*, **161**, 293-303.

17. SOKOLOFF, B., EDDY, W. H., BEAUMONT, J., WILLIAMS, J., & POWELLA, R. 1955. Effect of Ascorbic Acid and Glucoascorbic Acid on Nucleic Acids in Tumor Tissue. *Cancer Research*, **15**, 147-50.

18. ———, SAELHOF, C. C., & BEACH, J. 1955. Glucose Antagonists in Experimental Cancer. *AMA, Arch. Path.*, **59**, 729-32.

19. WICK, A. N., DRURY, D. R., & MORITA, T. N. 1955. 2-Deoxyglucose—A Metabolic Block for Glucose. *Proc. Soc. Exp. Biol. Med.*, **89**, 579-82.

20. WOODWARD, G. E., & HUDSON, M. T. 1954. The Effect of 2-Desoxy-D-glucose on Glycolysis and Respiration of Tumor and Normal Tissue. *Cancer Research*, **14**, 599-605.

Growth, 1956, **20**, 275-293.

ALLOMETRIC GROWTH OF THE DARK CORNISH FOWL[1]

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Variation in body form, or conformation, is of significance in diverse biological disciplines. The development of quantitative measures of conformation and the determination of genetic and environmental sources of variation in conformation are related problems that have received considerable attention. The study of changes in conformation of the individual during growth has also been the object of numerous investigations.

The body form or shape of an individual at any stage of development is a function of the relative proportion of the various parts and organs to each other and to the organism as a whole. D'Arcy Thompson (1917) pointed out that all but the simplest organisms reach their adult form by differential growth in different directions. Variation in conformation is the result of differences in the relative growth rates of the various dimensions and of duration of growth. Lerner (1939) emphasized that attention should be centered on the processes leading to the assumption of the specific form, and suggested the study of relative growth, or allometry, as a useful technique in studies of conformation.

Comprehensive and critical reviews of the vast literature relating to allometric growth have been completed by Reeve and Huxley (1945), Medawar (1945) and Richards and Kavanagh (1945). In this paper reference is limited to those publications relevant to this investigation.

The basic allometric relation is expressed by the equation

$$y = bx^a \qquad (1)$$

in which y represents a part, x another part or the whole, and "b"

[1] Publication approved by Directors of Louisiana and Nevada Agricultural Experiment Stations.
[2] Deceased, 1953.

and "a" are constants. The equation is either a parabola or hyperbola, depending on whether "a" is positive or negative. In logarithmic form the equation becomes

$$\log y = a \log X + \log b \qquad (2)$$

or

$$Y = K + aX \qquad (3)$$

where $Y = \log y$; $X = \log x$; and $K = \log b$.

In logarithmic form the allometric equation represents a straight line with slope "a" and Y intercept "K."

The allometry concept has evolved from several widely divergent areas of investigation of growth and form. There is, consequently, considerable confusion in the literature concerning many of the inherent problems of the allometry concept. Several difficulties relevant to this investigation are discussed briefly. Reeve and Huxley (1945) and Richards and Kavanagh (1945) present a comprehensive analysis.

Suggestions for a standard terminology and symbolism do not appear adequate, and have not been universally accepted. Kavanagh and Richards (1942) have shown that the data from most allometric studies may be classified into one of five types. These are:

Type A. Measurements of two variables, X and Y, are taken from time to time on the same individual and constitute the entire set. At each instant of time, t, each of the quantities X and Y has a definite value; each is a function of time: $X = f(t)$; $Y = g(t)$. These may be taken as the parametric equations of a curve in the strict sense. The points are determined by their coordinates, their order being determined by the values of t to which they correspond. The curve is continuous, although the slope may vary, and, when measurement error is negligible, the points all lie exactly upon it. Any deviation beyond that due to measurement error indicates that the allometric equation is not theoretically satisfactory. The most probable values for "b" and "a" can be shown to be those obtained by means of a least squares technique which accounts for measurement error in both variables.

Type B. A group of individuals as homogeneous as possible with respect to causative factors is selected, and one set of measurements is taken on each individual. Such data actually portray relative size rather than relative growth. There is no justification for consider-

ing the points to lie on a curve, or for considering "b" and "a" as growth constants if the allometric equation is fitted.

Type C. A series of Type B measurements is taken on the same group at successive intervals. In the usual analysis the arithmetic means $\overline{X}$ and $\overline{Y}$ are taken as the best representatives of the group of values at each stage. The successive points $(\overline{X}, \overline{Y})$ then form a curve in the sense described for Type A data. The curve is treated as the growth of an average individual, and the properties deduced from it are taken to represent the growth characters of the population. The finer characteristics of individual curves are obscured. Rarely does the growth of any one organism follow that of the hypothetical average.

In Type C data errors in corresponding $\overline{X}$'s and $\overline{Y}$'s are correlated. Hence, the fitting technique must take account of errors in both variables and the correlation between errors in the pairs $(\overline{X}, \overline{Y})$. Kavanagh (1941) has developed a "least quadratics" technique.

Type D. Measurements are made on a group of organisms without regard to differences between developmental stages of the individuals, and a two dimensional distribution of all the data is studied. In this type of data it is difficult or impossible to assign biological interpretation of the constants obtained from fitting the allometric equation. The strict curve relation between X and Y does not exist; the correlation type usually does.

Type E. A set of Type A curves is prepared for a group of individuals. These data permit the study of growth curves of individuals, and mathematical expressions may be deduced from them to express characteristics of these actual curves.

Very little data have been published in this form. The development of the theory and methods of fitting Type E data must await the collection of additional data and a knowledge of the utilization of the analysis. Clearly, it is with this type of analysis that the allometric equation has its greatest potential value. It is important to explore this area of research in greater detail.

At present it appears more satisfactory to give a clear statement of the class of data and purpose of the analysis rather than a further elaboration of terminology.

Several mathematical aspects of the allometric equation have been considered by Kavanagh and Richards (1942). They concluded:

(1) The equation is dimensionally valid, "a" being the ratio of two specific growth rates (dy/ydt:dx/xdt) and, therefore, a pure number. When x is measured in U^i and y in V^j, the dimensions of b are U^{-ia} V^j. (2) The relation between the parameters observed by Hersh (1931, 1934), $B=Be^{-ra}$, is due to the mathematical treatment. (3) Contrary to Lumer's (1937) conclusions, whatever may be the curve of sigmoid growth followed by one of the variables, the other may also be sigmoid without invalidating the allometric formula.

The allometric equation ignores time relations of growth by relating the sizes of the different parts of the body to total size regardless of age. Several attempts have been made to relate allometric growth to laws of time growth of organs and organisms. Reeve and Huxley (1945) have examined these in detail and conclude that no law of allometry may be derived from laws of growth in time. This cannot, however, be accepted as either criticism or justification for the allometric formula.

Haldane (see Huxley 1932, P. 81) pointed out that if the parts of an organ (e.g. segments of a limb) show unequal constant differential growth ratios against a standard, then the whole organ cannot obey exactly the allometric law against the same standard and vice versa, since the sum of a number of expressions b_rX^{ar}, with different values of ^{a}r, cannot be identical with a single expression bx^a. Teissier (1934) argued that since the discrepancy is slight when the values of "a" are not very different, the theoretical difficulty is disposed of in practice. However, it is certainly important when the biological meaning is to be interpreted.

A serious and frequently neglected difficulty is the determination of the adequacy with which the data fit the allometric equation. Simple statistical methods are not available for testing the linearity of the relation when both are subject to measurement error. Three main types of deviations from the allometric equation have been observed. These are:

(1) A curvilinear trend due either to increasing or decreasing values of "a".

(2) Critical points marked by sudden changes of shape or discontinuities.

(3) Rythmical variations about an average trend.

Kidwell et al. (1952) found evidence of the first two types of devi-

ation in cattle. The available data appear too meager to warrant any generalization concerning deviations from allometry.

Although the allometric equation had been used previously (Snell, 1891; DuBois, 1878, 1914; Lapicque, 1898; Klatt, 1919), Huxley (1924) empirically discovered the allometric equation, and suggested it might express a general law of differential growth. Later (1932), in an attempt to establish a theoretical basis for the equation as a biological law, he derived the formula on the basis of assumptions about growth in general. A number of investigators (Robb, 1929; Twitty, 1930; Teissier, 1934, 1937) have postulated different hypotheses to account for the allometric equation as a fundamental biological law of growth, but none has withstood critical analysis. It must be concluded that no satisfactory theoretical basis has yet been found for simple allometry. The allometric equation must be considered as nothing more than a statistical expression and not an intrinsic law of growth. The parameters cannot be given a physiological interpretation. However, the almost universal applicability of the equation to relative growth data cannot be overlooked. The equation may be considered as a measure of the resultant process or processes affecting growth, and as such may be considered a law.

This investigation was designed to determine:

(1) The adequacy of the allometric equation to describe the relative growth of certain dimensions in the Dark Cornish fowl. This would also provide additional Type A data.
(2) An estimate of the parameters of the equation if it provided a satisfactory description of relative growth in the Dark Cornish fowl.
(3) An evaluation of the allometric equation as a means of providing a quantitative description of conformation and of changes in conformation during growth.
(4) An estimate of the heritability of the parameters, thereby determining whether specified changes in conformation might be accomplished by selection for appropriate values of the parameters of the allometric equation.

Materials and Methods

The birds used in this investigation were representative of the 1951 spring hatch from the Louisiana State University Dark Cornish flock. Five sires were pen mated to six dams each. Three dams, one in one group and two in another, failed to produce surviving chicks.

Females were trapped during the egg collection period and each egg identified. All normal eggs were incubated. The chicks were identified with individual wing bands at hatching. There were two hatches of approximately equal numbers; one on March 9 and the other on March 23. Egg collection, storage and incubation followed the normal laboratory procedures. The chicks were sexed and battery brooded and grown through ten weeks of age. Standard laboratory procedures were employed for brooding and growth; the sexes were separated in the brooders and batteries.

Eighty-three males and 101 females survived the ten weeks of experiment. Only three males and two females of those placed in the brooders died before the conclusion of the experiment. All of the 184 chicks completing the ten weeks contributed complete records. It appears that Baker's (1944) requirement that the number of birds remain nearly constant throughout the experiment has been satisfied.

Six measurements were taken on each chick at one week of age and at weekly intervals thereafter through ten weeks. This particular period was selected as approximately coinciding with a desirable market age and weight. The measurements include weight, keel length, tarsometatarsus length, body depth, head width, and head length. The birds were weighed and measured at the same time each week, but feed and water were not removed prior to weighing. Weights were taken to the nearest gram. The remaining measurements were taken to the nearest tenth of a centimeter with a small machinist's caliper.

Weight was taken as a measure of mass; keel length as a measure of anterior-posterior growth; tarsometatarsus and body depth as measures of dorsoventral growth of two important dimensions. There were two reasons for including the head measurements: (1) Head shape has been considered a point of conformation by poultry breeders as well as by breeders of other farm animals. (2) In a study of allometric growth in beef cattle, Kidwell et al. (1952) fitted the Type E relation to head width/heart girth and head length/heart girth data. Heritability of "a" and "K" for head width/heart girth was estimated at zero while heritability of "a" and "K" for head length/heart girth was estimated at near unity. Although no physiological explanation of these differences is apparent, an obvious and important result is that changes in head shape relative to size (heart girth) result from changes in relative growth of head length only. It is of interest to

examine these relations in the Cornish fowl. A measure of body width would have been highly desirable. The available time and equipment precluded a satisfactory width measure in this study.

On the basis of studies with poultry (Lerner 1943) and cattle (Kidwell et al. 1952) it was concluded that weight is not a satisfactory measure to total size for studies of growth and form. Keel length and tarsometatarsus length which are subject to small measurement error and measure two important dimensions were selected as standards with which to compare the remaining measures. The requirements of the investigation were best met by a Type E analysis. In order to estimate graphically whether the data could be represented by the allometric equation a random sample of birds was drawn and for each a double logarithmic plot of the five remaining measures against keel length and tarsometatarsus length was made. Visual examination indicated a straight line might fit the plotted points within errors of measurement. The plots for these individuals were then fitted to the allometric equation and the parameters used to construct the fitted line. In each instance the perpendicular distance from the point to the fitted line was within the limit of measurement error. Although this is clearly recognized as a crude test, it seems reasonable to conclude that the data might be represented by the allometric equation. Five place logarithms (base 10) of the original measurements were fitted to the allometric equation.

If the logarithmic form is fitted it is necessary to consider the question of weighting, since deviations from the parabola are not equal to deviations from the linear logarithmic form. Kenny (1947) states the discrepancy usually does not affect the fit seriously. Kavanagh and Richards (1942) drew evidence from the data of Needham (1934) and Teissier (1934) to show that there would be little difference between the correctly weighted and unweighted methods of treatment of the logarithmic form. For the purpose of this study, the increased precision would not justify the additional labor of weighting.

Kavanagh and Richards (1942) have shown that for the individual curves of Type E the most probable values of "b" and "a" are those obtained by use of a least squares technique that accounts for measurement error in both variables. Deming (1938) presented a comprehensive discussion of least squares technique accounting for error in both variables, including the application to both forms of the allometric equation. Wald (1940) examined a simple method of fitting a

straight line when both variables are subject to error. A modification of Wald's method was presented by Bartlett (1949). A method was also suggested by Kenny (1947). Bartlett's method was not available at the time this experiment was designed, and it was intended to use Kenny's (1947) method, as it had been used by Kidwell et al. (1952). Since Bartlett's method is much less laborious, a sample of ten birds was selected and the parameters determined by both methods. Differences between them were significant at the five percent level. It is possible that the observed significant difference was itself a sampling error. Certainly the authors are unable to determine which of all available methods is theoretically most satisfactory. These data were fitted by Kenny's method, which minimizes the sum of the squares of the distances from the plotted points perpendicular to the fitted line. The formulae for "a" and "K" are:

$$a = \frac{(\sigma_y^2 - \sigma_x^2) \pm \left\{(\sigma_y^2 - \sigma_x^2) + 4r_{xy}\ (\sigma_{xy})^2\right\}^{1/2}}{2\ r_{xy}\ \sigma_{xy}} \qquad (4)$$

$$K = \overline{Y} - a\overline{X} \qquad (5)$$

where σ_x^2 and σ_y^2 are the variances of X and Y; r_{xy} is the product-moment correlation, and σ_{xy} the co-variance between X and Y.

Results and Discussion

Weekly means of each measurement for each sex are presented in Table 1. These data are presented graphically in Figure 1. An early sexual dimorphism is clearly evident in all dimensions, but is smallest for the head measurements. These data may be considered as representative of normal growth of the Dark Cornish through ten weeks of age.

Means and standard errors of a and K are shown in Table 2. Sex differences were determined by analysis of variance. The data for both sexes were combined into a single estimate where no significant differences were found. An illustration of the data is provided in Figure 2, which presents double logarithmic plots of the data of a randomly selected female, the fitted equation and curve.

No sex difference was observed for either the weight/tarsometatarsus or the weight/keel length relations, although there is a marked sex difference in weight throughout development. It appears that the increase in weight relative to tarsometatarsus and keel length is the

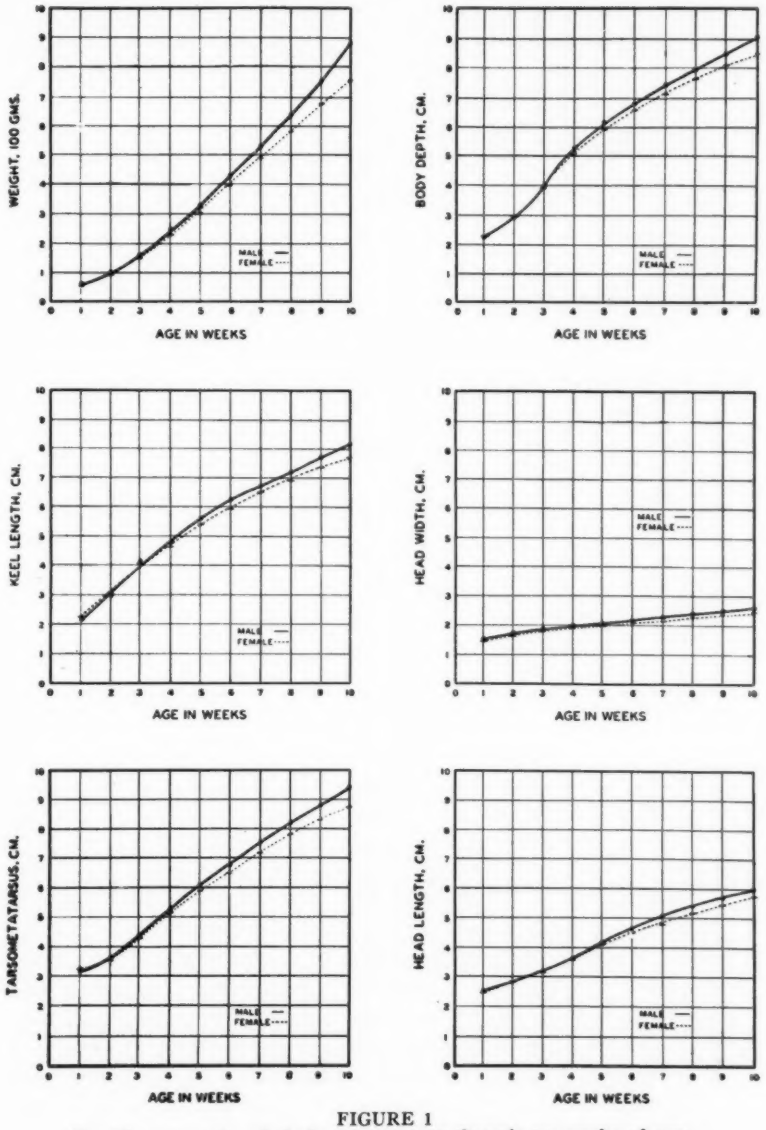

FIGURE 1
Growth curves of each body measurement through ten weeks of age.

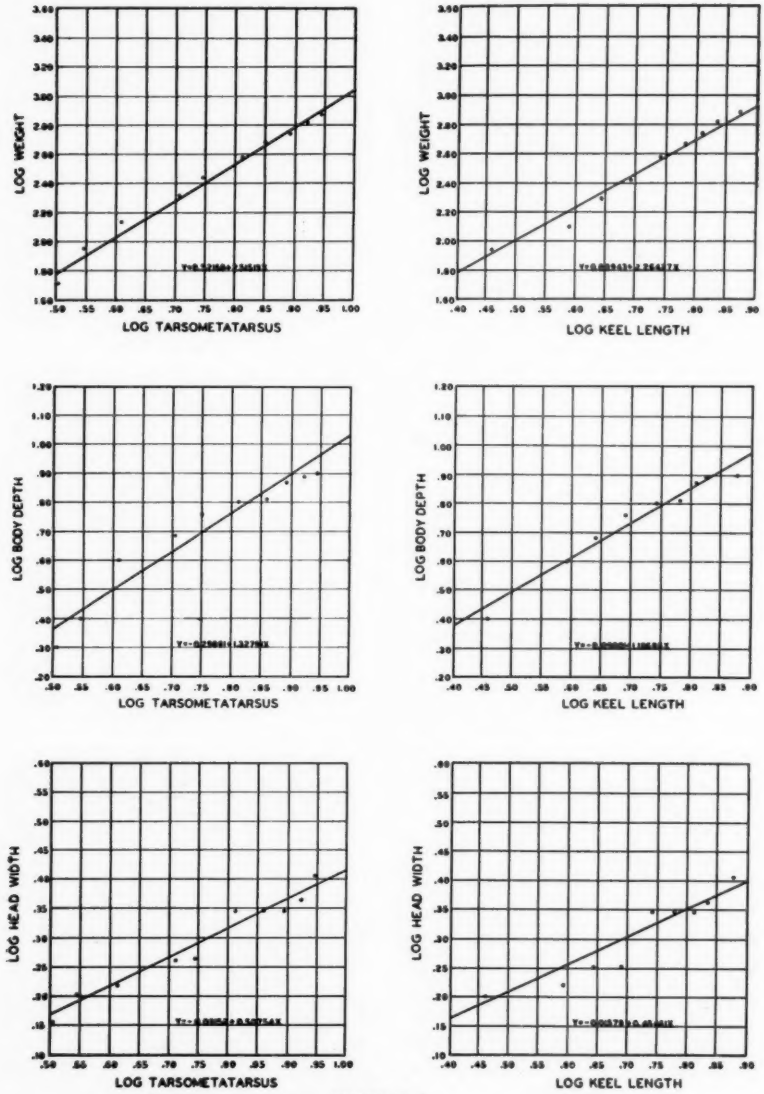

FIGURE 2
Double logarithmic plots of the allometric equation of a typical female.

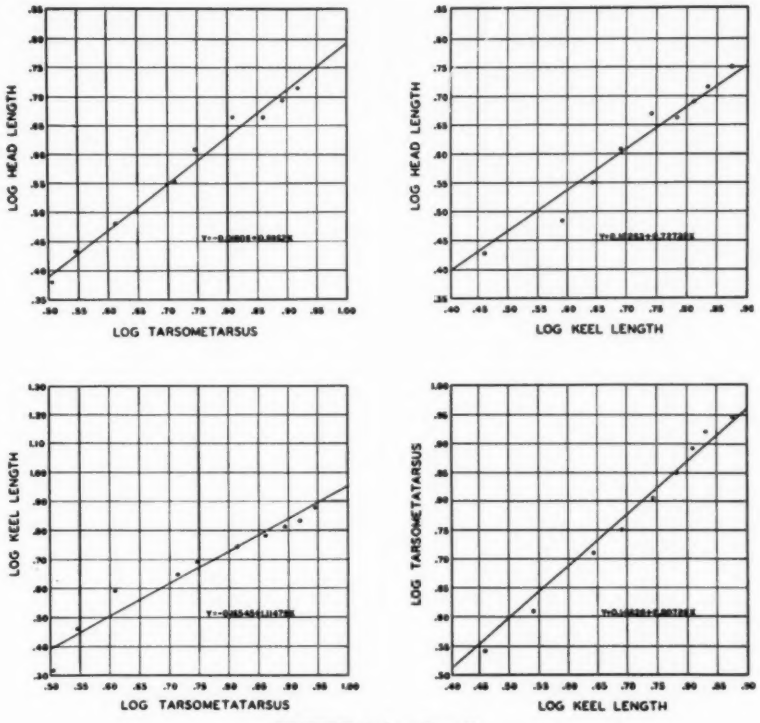

FIGURE 2 (continued)

same for both sexes. It also follows that the sexes have the same weight relative to tarsometatarsus or keel length; i.e., males and females of equal tarsometatarsus or keel length will be of equal weight.

The body depth/tarsometatarsus relation is of interest in that there is a sex difference for "a" but not for "K." "K" is the Y intercept or the value of Y when X equals zero. Since X is the logarithm of the actual measure, it would have a value of zero when the actual measure had a value of one. This would correspond to a stage of development considerably earlier than hatching. Kidwell et al. (1952) showed that, with cattle data, extrapolation into embryonic development is not valid for purposes of biological interpretation. It is likely unwise to attempt interpretation in the present instance.

It is also true that the lines for males and females intersect when X equals zero. It appears that at hatching females are deeper of body, relative to tarsometatarsus and that their body depth relative to tarsometatarsus increases at a greater rate than that of males.

TABLE 1
Week by Sex Means of Six Body Measurements Taken on 83 Male and 101 Female Dark Cornish Chicks.

Week	Sex	Weight gm	Keel Length cm	Tarso-metatarsus cm	Body Depth cm	Head Width cm	Head Length cm
1	Male	56.42	2.12	3.16	2.29	1.52	2.50
1	Female	55.31	2.21	3.17	2.32	1.49	2.49
2	Male	99.42	2.99	3.64	2.98	1.66	2.74
2	Female	97.69	3.09	3.62	2.99	1.65	2.75
3	Male	155.25	4.13	4.40	3.96	1.80	3.14
3	Female	152.37	4.09	4.31	3.93	1.77	3.13
4	Male	249.13	4.83	5.24	5.36	1.95	3.66
4	Female	236.41	4.72	5.10	5.12	1.88	3.63
5	Male	329.04	5.62	6.10	6.20	2.03	4.20
5	Female	309.64	5.42	5.89	5.91	1.97	4.06
6	Male	430.07	6.20	6.77	6.85	2.17	4.69
6	Female	395.82	5.96	6.49	6.61	2.11	4.55
7	Male	530.06	6.76	7.51	7.41	2.29	5.04
7	Female	487.51	6.49	7.15	7.18	2.20	4.88
8	Male	639.84	7.18	8.22	7.94	2.38	5.38
8	Female	584.26	6.92	7.78	7.67	2.28	5.18
9	Male	757.04	7.73	8.80	8.52	2.47	5.71
9	Female	678.47	7.36	8.28	8.14	2.35	5.49
10	Male	886.57	8.21	9.39	9.00	2.57	6.00
10	Female	758.66	7.76	8.74	8.52	2.42	5.76

Sex differences in "a" and "K" were observed for the body depth/tarsometatarsus relation. Simultaneous solution of the two equations indicates the lines intersect when keel length has a value of 5.1 cm., which corresponds to approximately four weeks of age. It appears that at hatching males have greater body depth relative to keel length, but that females have a higher relative growth rate. At about four weeks of age the two sexes have the same body depth relative to keel length; thereafter the females develop greater depth relative to keel length than the males.

A consequence of the two foregoing relations is that at normal market size females are deeper of body, relative to both tarsometatarsus and keel length, than males and thus "blockier" in appearance; i.e., body depth and length are more nearly equal. Data on capons are not available, but should their growth pattern be intermediate, it would explain in part their general desirability.

TABLE 2
Means and Standard Errors of a and K for Each Measure with Tarsometatarsus and Keel Length as Standards.

		Tarsometatarsus		Keel Length	
		a	K	a	K
Weight		2.47555 ± .01260	0.57142 ± .00482	2.12631 ± .01261	0.95728 ± .00990
Body Depth	M[1]	1.26429 ± .00427	0.24517 ± .00604	1.07938 ± .00842	—0.02682 ± .00533
	F	1.29892 ± .01146		1.11500 ± .01032	—0.05191 ± .00885
Keel Length	M / F	1.17330 ± .00551	—.19010 ± .00421	———	———
Tarsometatarsus		———	———	0.85705 ± .00400	0.15930 ± .00305
Head Width		0.46136 ± .00332	—.04857 ± .00276	0.39082 ± .00349	0.02886 ± .00259
Head Length	M	0.83069 ± .00454	.03367 ± .00272	0.71245 ± .00435	0.10998 ± .00598
	F	0.84556 ± .00437			

[1] Estimates for males and females are shown separately only in instances where a significant sex difference exists.

The keel length/tarsometatarsus and tarsometatarsus/keel length relations require no further comment. Actually, they are related, keel length being the independent variate in one case and the dependent variate in the other.

No sex differences were observed in the head width/tarsometatarsus or head width/keel length relations, nor was there any sex difference in the head length/keel length relation. It appears, however, the females have a slightly greater rate of growth of head length relative to tarsometatarsus than males. In cattle, Kidwell et al. (1952) found no sex difference in the head length/heart girth relation, but males had a relatively greater rate of growth of head width relative to heart girth. Insofar as heart girth of cattle and tarsometatarsus of the Dark Cornish fowl can be considered as measures of size, these relations have the same net result. The ratio of head length to head width is smaller for males than for females of the same size.

This experiment was designed to provide estimates of the heritability of the parameters. The small number of sires, necessitated by limited time and facilities, gives rise to large sampling errors. Consequently, the estimates cannot be considered as extremely accurate. However, they provide a good indication of the relative order of importance of genetic and environmental sources of variation. The data were adjusted for sex differences where appropriate before heritability estimates were made. The heritability estimates are presented in Tables 3 and 4.

The heritability estimates were obtained by the method of analysis of various components described by King and Henderson (1954). This method provides heritability estimates in the narrow sense; i.e., the ratio of the additive genetic variance to the total or phenotypic variance. The method provides two estimates of heritability, one based on the sire and one on the dam component of variance.

In the present population deviations from random mating were so slight as to be negligible. Consequently, the heritability estimates were not adjusted for deviations from random mating.

Although there is considerable variation, the estimates are generally low. This is at variance with the observations of Kidwell et al. (1952) with cattle, but in agreement with those of Lerner (1943) with a Leghorn X Rock Cross. Lerner considered the "a" values of the tarsometatarsus/weight relation. His method of estimating "a" was different from that used in this study. At the end

of four generations of progeny-test selection involving 320 offspring, the high "a" and low "a" lines did not differ from each other. Lerner concluded that the genetic variability of the relative growth pattern was either non-existent or so low as to preclude its demonstration by selection in opposite directions. The low heritability estimate found in this population indicates that selection would be ineffective.

TABLE 3
Heritability Estimates of a and K for Tarsometatarsus with Indicated Measures.

Estimate from	Weight		Body Depth		Keel Length		Head Width		Head Length	
	a	K	a	K	a	K	a	K	a	K
Sires	.105	—.071	.146	.018	.141	.191	—.090	.060	.055	.385
Dams	.039	.764	.561	.471	.038	—.375	.076	.099	.028	.449

TABLE 4
Heritability Estimates of a and K for Keel Length with Indicated Measures.

Estimate from	Weight		Body Depth		Tarso-metatarsus		Head Width		Head Length	
	a	K	a	K	a	K	a	K	a	K
Sires	—.065	—.019	.129	.156	.063	.047	.434	.135	.278	—.184
Dams	—.026	—.080	—.047	.048	.245	.069	.049	.293	.106	.613

The beef cattle data lead to the conclusion that relations involving measure of skeletal growth might be fairly highly heritable, while those involving muscular and fat growth would not. These data indicate low heritabilities of the parameters of all relations.

An established explanation of these results is not immediately evident. Clearly, differences in relative proportions at any stage of development among domestic fowl exist. In fact, there was considerable variation in this population. These differences must have resulted from differential growth in different directions. The allometric equation has adequately described these patterns for the dimensions studied. Recent changes in the relative proportions of market broilers and fryers clearly indicate a genetic source of variation. The ease and rapidity with which conformation has been changed indicates a large proportion of the variance is due to additive gene action.

Several possible explanations of the low heritabilities exist: (1) The relationships studied are not satisfactory for describing conformation. (2) The genetic variance is largely non additive, i.e., due to dominance and epistatic deviations. (3) The heritability is real but

low so that the small sample (sires) and large sampling error precluded demonstration. (4) Differences in relative growth rates, and hence differences in proportions, are largely the result of environmental differences. The data do not permit a critical evaluation of these theories. Other explanations might exist.

Hersh (1931, 1934) observed the relation $b = Be^{-ra}$, where "b" and "a" are parameters of the allometric equation; B and r are constants, and e is the base of the natural logarithm. Lumer (1936) showed this relation to be the necessary and sufficient condition that the curves all pass through a common point. Kavanagh and Richards (1942) studied Hersh's data and pointed out that because of the unit of measure and because the organs measured were all of roughly comparable size, the points were grouped in a fairly small area of the plane compared with the distance to the line $X = O$. Since "K" is the value of Y when $X = O$, determination of "K" from the data represents extrapolation a considerable distance beyond the data and an approximation to Lumer's condition that all curves pass through a common point. Since the distance to the line $X = O$ depends on the unit of measure of X, it can be moved closer, or even into the group of points by taking a large unit of X and as the degree of extrapolation diminishes, the relation between the parameters diminishes.

In the data of Kidwell et al. (1952) the small unit of measure of X resulted in a close approximation to Lumer's condition that all curves pass through a common point. Consequently, "K" was found to be largely an inverse of "a," most of the variation in "K" being merely a magnified inverse of the variation in "a." This was clearly illustrated in the close similarity of heritability estimates of corresponding values of "a" and "K"; and by the estimates of, or closely approaching, —1.0 of phenotypic, genetic, and environmental correlations between corresponding values of a and K. Contrary to the conclusions of Kavanagh and Richards (1942) that, since the value of b depends on the unit of measure no unique biological significance or interpretation may be attached to it, Kidwell et al. (1952) concluded that, in a type E analysis employing a relatively small unit of measure of X, "a" and "K" are measures of the same biological phenomenon. It is necessary that the same unit of measure be used for different values of "K" to be comparable.

In these data the relatively small unit of measure of X and the

similar size of the various dimensions results in extreme extrapolation and close approximation to Lumer's conditions. The general similarity in heritability estimates of corresponding values of "a" and "K" illustrate the expected automaticity.

Summary and Conclusions

One hundred and eighty-four Dark Cornish chicks were hatched and grown through ten weeks of age at the Louisiana State University Poultry Laboratory. Standard conditions of hatching, brooding, and growing prevailed. Weight, body depth, keel length, tarsometatarsus length, head width and head length were measured at one week of age and weekly thereafter through ten weeks. Logarithms of the remaining five measurements were plotted against keel length and tarsometatarsus length in a Type E allometric analysis. It was concluded that the allometric equation was theoretically satisfactory to describe the relative growth of these dimensions. Sexual dimorphism was noted and discussed for several of the relations.

Estimates of the heritability of "a" and "K" were obtained from an analysis of variance components. The estimates were generally low. Several possible reasons are presented, but critical analysis is not possible. The automaticity of the similarity of heritability estimates of corresponding values of "a" and "K" is noted and discussed.

Acknowledgment

This experiment was designed and the data collected while the senior author was on the Animal Industry Staff and the junior author a graduate assistant in Poultry Industry at the Louisiana State University. Shortly after the data were collected the senior author assumed his present position and the junior author succumbed, a victim of luekemia. In the long course of analysis of the data and preparation of the manuscript the senior author has become indebted to many people. Sincere appreciation is hereby expressed to Professor C. W. Upp of Louisiana State University for encouraging the undertaking, making available the birds, feed, facilities, and routine care and assisting with the preparation of the manuscript. Mrs. Dorothy Wilson, Secretary in Animal Industry at Louisiana State University, made the logarithmic transformations. The University of Nevada Research Committee made $250.00 available for I.B.M. treatment of the data. Mr. Philip N. Ternan, Graduate Assistant

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References

Baker, G. A. 1944. Weight-Growth Curves. *Poultry Science,* **23**, 83-90.

Bartlett, M. S. 1949. Fitting a Straight Line When Both Variables Are Subject to Error. *Biometrics,* **5**, 207-212.

Deming, W. E. 1938. Some Notes on Least Squares. U. S. Department Agriculture, Washington.

Dubois, E. 1898. Uber die Abhangigkeit des Hirngewich les Von der Korpergross bei den Saugethieren. *Arch. f. Anthropol.,* **25**, 1-28.

Hersh, A. H. 1931. Facet Number and Genetic Growth Constants in Bar-eyed Stocks of Drosophila. *Jour. of Exp. Zool.,* **60**, 213-48.

———. 1934. Evolutionary Relative Growth in the Titanotheres. *Amer. Nat.,* **68**, 537-61.

Huxley, J. S. 1924. Constant Differential Growth-Ratios and Their Significance. *Nature,* **114**, 895-96.

———. Problems of Relative Growth. 276 P. Methuen and Co., London.

Kavanagh, A. J. 1941. Note on the Adjustment of Observations. *Ann. Math. Stat.,* **12**, 111-14.

Kavanagh, A. J., & Richards, O. W. 1942. Mathematical Analysis of the Relative Growth of Organisms. *Proc. Roch. Acad. Sci.,* **8**, 150-74.

Kenny, J. F. 1947. Mathematics of Statistics. 2nd Ed. Part I, 260 pp. D. Van Nostrand, New York, New York.

Kidwell, J. F., Gregory, P. W., & Guilbert, H. R. 1952. A Genetic Investigation of Allometric Growth in Hereford Cattle. *Genetics,* **37**, 158-74.

King, S. C., & Henderson, C. R. 1954. Variance Component Analysis in Heritability Studies. *Poultry Science,* **33**, 47-54.

Klatt, B. 1919. Zur Methodik Vergleichender Metrischer Untersuchunger, Besonders des Herzgewichtes. *Biol. Z61,* **39**, 406-12.

Lapicque, L. 1898. Sur la Relation du Poids de l'encephalc au boids due Corps. *Compt. Rend. Soc. Biol., Paris,* **50**, 62-64.

Lerner, I. M. 1939. Allometric Studies in Poultry. Proc. 7th Worlds Poultry Congress, Cleveland, 85-88.

———. 1943. The Failure of Selection to Modify Shank-Growth Ratios of the Domestic Fowl. *Genetics,* **28**, 80-81.

Lumer, H. 1936. The Relations Between b and K in Systems of Relative Growth Functions of the Form $y = bX^K$. *Am. Nat.,* **70**, 188.

Lumer, H. 1937. The Consequences of Sigmoid Growth for Relative Growth Functions. Growth, **1**, 140-54.

Medawar, P. B. 1945. Size, Shape and Age. Essays on Growth and Form 157-87, Clarenden Press, Oxford.

Needham, J. 1934. Chemical Heterogamy and the Ground Plan of Animal Growth. *Biol. Revs.,* **9**, 79-109.

Reeve, E. C. R., & Huxley, J. S. 1945. Some Problems in the Study of Relative Growth. Essays on Growth and Form. 121-56. Clarendon Press, Oxford.

Richards, O. W., & Kavanagh, A. J. 1945. The Analysis of Growing Form. Essays on Growth and Form: 188-230. Clarendon Press, Oxford.

Robb, R. C. 1929. In the Nature of Hereditary Size Limitation. II. The Growth of Parts in Relation to the Whole. *Brit. J. Exp. Biol.,* **6**, 313-24.

SNEDECOR, G. W. 1946. Statistical Methods. 4th Ed. xvi+485 pp. Iowa State College Press.

SNELL, O. 1891. Die Abhangigkeit des Hirngewichtes von dem Korporgewicht und der Geistgen Fahigkeiten. *Arch f. Psychol. U. Nervenkrank.*, **23**, 436-46.

TEISSIER, G. 1934. Dysharmonies et Disconuites dans la Croissance. Actual. Sci. Ind. Nr. 95, Paris, 3-39.

————. 1937. Les Lois Quantitatines de la Croissance. Actual. Sci. Ind. Nr. 455, Paris, 47 pp.

THOMPSON, D. W. 1917. On Growth and Form. 793 pp. Cambridge Univ. Press.

TWITTY, V. C. 1930. Regulation in the Growth of Transplanted Eyes. *J. Exp. Zool.*, **55**, 43-52.

WALD, A. 1940. The Fitting of Straight Lines if Both Variables Are Subject to Error. *Ann. Math. Stat.*, **11**, 284.

Growth, 1956, **20**, 295-313.

THE EMBRYONIC DEVELOPMENT OF THE THYROID GLAND IN THE FROG, *HYLA BRUNNEA*[1]

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Our knowledge of the origin and development of the thyroid gland in anuran amphibians is primarily derived from studies on common European and American species of the genus *Rana*. Early contributions to the subject are contained in the works of Müller (1871) and Maurer (1888). Gudernatch's discovery (1912) that the thyroid plays a leading role in amphibian metamorphosis aroused interest in thyroid physiology and also led to renewed study of the embryology and morphology of the gland. The investigations of Sklower (1925), Etkin (1930, 1936), Clements (1932), D'Angelo and Charipper (1939), D'Angelo (1941), Steinmetz (1952, 1954), and others have clearly demonstrated a close relationship between the developmental history of the thyroid and the metamorphic pattern in *Rana*. Similar, though less extensive studies, have been carried out on representatives of other genera such as *Bufo* (Allen, 1919; Morita, 1932) and *Pseudacris* (Etkin, 1936). In addition certain less common anurans, whose life history is marked by the absence of a tadpole stage, have attracted attention. Two such genera in which studies of the development of the thyroid have been made are *Eleutherodactylus* (Lynn, 1936, 1942) and *Arthroleptella* (Brink, 1936, 1939).

The genus *Hyla* is a large group of amphibians extensively distributed in tropical and temperate regions. Its members show rather wide variations in breeding habits, in choice of egg-laying sites, and in larval structure. Many species lay their eggs in ponds and pass through a life-history essentially like that of *Rana pipiens*. Others,

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however, build mud basins at the edges of streams or pools and lay the eggs in these small muddy cups. The tadpoles of these species have greatly enlarged gills, apparently in adaptation to the low oxygen supply. One species, *H. uranochroa,* lays its eggs out of the water, on leaves, and the tadpoles wriggle into the water after hatching.

In the American tropics there are a number of hylas which have adopted still another breeding site, laying the eggs in the water held in the leaf-bases of broad-leaved epiphytic plants (bromeliads) growing on tree branches. The tadpoles which live in this restricted space are modified by having flattened bodies, unusually long tails, and greatly reduced pigmentation. Their gills are small and the animals seem to get their oxygen directly from the air. The larval teeth and jaws are modified in relation to the fact that the tadpoles feed almost exclusively on frog eggs; in most cases, apparently, those of their own species. The details of the metamorphic pattern in these forms have not been worked out but it seemed likely that metamorphosis is considerably modified as compared with that of pond-dwelling forms and that an investigation of the metamorphic pattern and its relation to thyroid function would be of interest.

The present study concerns one of these bromeliad-inhabiting species. An account will be given of the origin and development of the thyroid gland and an attempt will be made to ascertain the relationship between thyroid activity and the principal events of metamorphosis.

Materials and Methods

The material for this study was selected from a large series of embryos and larvae of *Hyla brunnea* (Gosse) collected in Jamaica, B.W.I. by W. G. Lynn during the period from July, 1952 to September, 1953. Specimens representing very early developmental events (cleavage, gastrulation, and neural groove formation) have been eliminated from consideration since the origin of the thyroid occurs at the neurula stage. The specimens utilized were first classified, eight stages ranging from the neurula to the newly metamorphosed frog being defined on the basis of observation and measurement of various external morphological features such as body, tail, hind leg and fore leg length. These measurements were made to the nearest tenth of a millimeter by use of calipers. The various stages will be described later.

All specimens had been fixed in a 1:1 mixture of Bouin's fluid and cellosolve. For each stage 5 to 10 specimens were selected for sectioning. These were cleared in benzene and embedded in paraffin. Both sagittal and transverse serial sections were cut at 10 micra. They were stained with Mallory's triple stain.

The sectioned material was subjected to detailed study, special attention being given to the origin of the thyroid anlage, the origin and development of follicles, and the histological changes exhibited throughout development. In addition to the observational study, measurements were made for several features.

Camera lucida drawings at a magnification of 560 X were made from the central sections of both the right and the left thyroid of each animal. A direct count of the number of follicles in the central section was made for each gland and the average height of the follicular epithelium and the longest and shortest diameters of the follicles were measured. The thyroid area, the area of the follicular epithelium and the colloid area in the median section of each gland were determined from the camera lucida drawings by use of a planimeter. The volume of the entire gland was calculated by multiplying the area (median section) of the gland by the dimension obtained from the number of sections which showed the presence of thyroid tissue. Using the formula devised by Uhlenhuth (1938), the percentage of the colloid was estimated.

A summary of the numerical data is given in Table I (p. 298). Stage I, which covers the period before the division of the thyroid into two lobes, is omitted from the summary. Stage II is included but the numerical data are derived from only the more advanced specimens in which the thyroid follicles are definitely distinguishable. Stages IV and V have been sub-divided into Sub-stages "A" and "B" in order to give a more detailed representation of the gradual development of the thyroid gland. Recorded data are averages obtained from measurements on 5 to 10 animals for each stage.

Observations

As previously noted, the large series of embryos and larvae used for this study was divided into eight groups or "Stages", each group representing a range of successive developmental periods. The Stages may be characterized as follows:

I. Late embryonic stage.—This stage covers the embryonic period

from closure of the neural tube to formation of a well-defined tail bud. Prominent external features are the ventral suckers, the stomodeal invagination, and, in more fully developed specimens, the external gills. Although the external morphology is the basis for definition of the stages, certain internal features, as revealed in the sectioned specimens, are worthy of note. The optic vesicles are in the process of transforming into optic cups during this period. The lens placodes are present but are still in continuity with the surface ectoderm. The otic vesicles, however, have completely separated

TABLE I
Summary of Numerical Data.

Stage	II	III	IV A	IV B	V A	V B	V	VII	VIII
Body length (mm)	2.5	5.1	7.5	9.1	10.2	15.9	14.0	12.5	11.5
Tail length (mm)	7.5	14.6	19.8	26.6	30.4	31.2	26.3	5.4	1.5
Hind leg length (mm)	—	—	0.8	2.5	7.6	17.8	21.1	17.6	18.5
Fore leg length (mm)	—	—	—	—	—	—	10.5	9.6	9.1
Area of median section of entire gland ($mm^2 \times 10^{-3}$)	0.7	2.1	7.5	13.0	45.2	82.0	86.0	59.0	64.0
Volume of entire gland ($mm^3 \times 10^{-4}$)	0.5	2.4	11.5	49.4	162.8	381.8	407.0	179.8	227.5
Number of foll. per median sect.	0.7	2.2	4.4	5.6	9.6	12.8	15.9	13.3	16.8
Longest diam. of follicles ($mm \times 10^{-3}$)	21.0	30.0	42.0	66.0	89.0	118.0	129.0	110.0	112.0
Shortest diam. of follicles ($mm \times 10^{-3}$)	17.0	20.0	29.0	42.0	58.0	85.0	85.0	68.0	73.0
Height of follicular epith. ($mm \times 10^{-3}$)	4.0	5.0	6.4	7.0	10.0	12.0	14.0	20.0	9.8
Area of follicular epith. per median sect. ($mm^2 \times 10^{-4}$)	1.0	6.9	27.0	63.0	223.0	280.0	340.0	216.0	240.0
Area of colloid per median sect. ($mm^2 \times 10^{-4}$)	0.2	3.0	16.0	55.0	150.0	330.0	270.0	153.0	302.0
Colloid percentage per median section	16.0	30.0	39.0	45.8	40.6	54.0	44.7	41.5	57.0

from the surface ectoderm by the end of this stage. Considering these features, Stage I may be said to correspond to Stages 16 to 18 of *Rana sylvatica* as defined by Pollister and Moore (1937). In *Hyla brunnea* the total length of the embryo at this period is 2.0 to 3.0 mm.

II. Stage of resorption of external gills.—During this stage the resorption of the external gills takes place and the internal gills become established. The ventral suckers are lost, the larval mouth with its chitinous teeth is formed, and a marked growth of the tail begins. Larvae ranging in total length from 4.0 to 13.0 mm fall into this stage. The body length of these larvae is only 1.0 to 3.0 mm, however, while the tail length ranges from 3.0 to 10.0 mm. This group of specimens

corresponds to Pollister and Moore's (1937) Stages 19 through 23 for *Rana sylvatica*.

III. Early larval stage.—Larvae in which the external gills have been resorbed but no hind limb buds have yet appeared constitute Stage III. Animals representing this stage range in body length from 4.0 to 6.0 mm and have tails ranging from 11.0 to 18.0 mm.

IV. Hind limb bud stage.—All larvae with hind limb buds up to 3.0 mm in length are included in this stage. It is clear, however, that during this "pre-metamorphic" stage, i.e. before there is any significant increase in rate of growth of the hind legs, there is a marked increase in the size of the body as a whole. Larvae of this group range from 7.0 to 11.0 mm in body length and from 11.0 to 30.0 mm in tail length. This stage has therefore been divided into two Sub-stages: A, consisting of larvae with body length of 7.0 to 9.0 mm and B, consisting of larvae with body length of 9.5 to 11.0 mm.

V. Stage of rapid hind limb growth.—Larvae of this stage have clearly-defined, jointed hind limbs ranging from 5.0 to 21.0 mm in length, while the body length is 11.0 to 20.0 and the tail length 26.0 to 35.0 mm. Since this stage, like the preceding one, covers a rather wide range of sizes, it also is divided into two Sub-stages: A, which includes animals with hind limbs of 5.0 to 15.0 mm in length and B, which includes those with hind limbs of 16.0 to 21.0 mm.

VI. Fore limb emergence stage.—In animals at this stage one or both fore limbs have emerged from the opercular cavity and the tail is still over 20.0 mm long. Hind limb growth continues in this stage, limb length ranging from 17.0 to 24.0 mm. The body length decreases slightly during this period, measuring 13.0 to 15.0 mm. The chitinous mouth furnishings of the larva are shed and the mouth form begins to approach that of the adult.

VII. Tail resorption stage.—Following Stage VI, the tail is resorbed quite rapidly. This is the last externally visible feature of the metamorphic pattern. Animals of Stage VII have the tail length reduced, the range now being 3.0 to 16.0 mm; the body length is 12.0 to 13.0 mm.

VIII. Post-metamorphic stage.—Recently metamorphosed individuals which still show a small tail stump (less than 3.0 mm) but have the typical frog mouth and tongue, represent this stage. The

body length is 10.0 to 12.0 mm, hind limbs 15.5 to 20.0 mm, and fore legs 7.5 to 10.0 mm.

Development of the thyroid

The thyroid anlage is identifiable in *Stage I* as a solid proliferation of highly pigmented entodermal cells from the pharyngeal floor immediately anterior to the bases of the hyomandibular pouches. The anlage projects posteriorly to about 5 to 10 micra in front of the pericardium, where it widens and assumes a compact spherical form. At this point it measures 45 to 55 micra in diameter. For a short time the connection with the pharynx is retained by means of a thin stalk consisting of entodermal cells which are identical with those of the pharynx and of the thyroid anlage (fig. 1). As the latter continues to migrate posteriorly there is also a general elongation of the pharyngeal region so that the thyroid soon loses its connection with the pharyngeal entoderm and comes to lie beneath the hyoid cartilages which are now in the process of differentiation. By this time the posterior part of the thyroid is in close proximity to the bifurcation of the ventral aorta. In most examples, the entire structure is still a heavily pigmented, compact aggregation of entodermal cells. In some specimens, however, the thyroid mass is a hollow structure with several layers of cells surrounding a small central cavity (fig. 2). This condition is apparently quite transient for it is not found at any later stage. At this early time of development the neighboring mesenchymal cells have come into close contact with the outside of the primitive thyroid tissue.

In younger specimens of *Stage II* the developing thyroid is observed in the process of dividing into right and left lobes. The almost spherical thyroid anlage begins to flatten horizontally, finally forming a median, bandlike, solid layer of highly pigmented tissue which is about 100 micra in length and 7.5 micra in width. This then splits so that eventually the right and left thyroid masses are connected by a thin strand of pigmented cells which traverses the copula ventrally.

In the thyroids of some of the more advanced specimens of Stage II the epithelial cells are organized into cords or are arranged in scattered clusters of cells held together by the surrounding mesenchyme. In a few regions the cells have separated, leaving channel-like spaces which, in some specimens, are filled with a faintly stained

substance. At this early stage then, there are indications of the presence of the first secretion of colloid. The epithelium, nevertheless, is still poorly defined. It appears to be undergoing a transition from squamous to cuboidal. The volume of the thyroid of this stage is approximately 0.5 x 10^{-4} mm^3.

By *Stage III* the thyroid lobes have assumed their definitive positions ventral to the lateral wings of the hyoid cartilages and dorsal to the genio-hyoid muscles. However, their shape is highly irregular; often the tissue is broken into two or three closely adjoining, but separate, epithelial masses (fig. 3) within each of which follicles can be observed. There is a great increase in vascularization and a pronounced reduction in pigmentation. The follicular epithelium is cuboidal and its nuclei are large and spherical. Mitotic figures are abundant at this stage and most of the follicles enclose compact masses of homogeneous colloid which is predominantly basophilic. The average volume of the gland is about 2.4 v 10^{-4} mm^3.

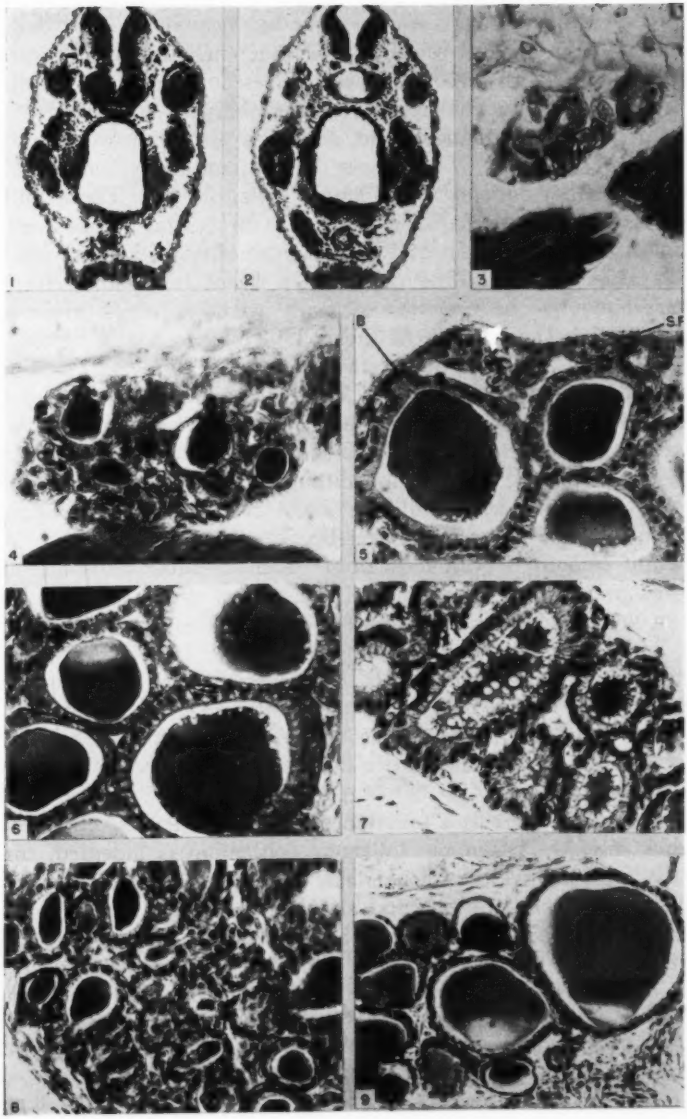
At *Stage IV*, when the hind limb buds appear, the thyroid bodies have become more regularly shaped structures which are separated from the hyoid cartilages only by a thin layer of connective tissue (fig. 4). The volume of the gland has greatly increased and measures about 11.5 x 10^{-4} mm^3. Pigmentation is lacking. Extensive vascularization is indicated by the presence of numerous blood sinuses. The thyroid lobes contain many definite follicles of various sizes. The follicular epithelium is higher than that of the preceding stages, although it still consists principally of cuboidal cells. In a few of the larger follicles the epithelium is low columnar with nuclei basal in position and quite large, filling at least half the cell body. Such follicles generally contain colloid with many peripheral vacuoles. The colloid is clearly basophilic, particularly in those follicles which contain a compact non-vacuolated colloid.

During *Stage V* there is a striking change in thyroid structure. The individual lobes have greatly increased in size, the volume now ranging from 162.8 to 381.8 x 10^{-4} mm^3. They are regular in outline and ellipsoidal in shape. In the more advanced embryos at this stage the thyroid bodies are frequently located further away from the hyoid cartilages than formerly so that they lie in the mesenchymal tissue beneath the hyoid apparatus. Some blood spaces are present, but on the whole, vascularization has decreased notably. The follicles are usually medium sized. In some regions of the follicular

PLATE I

Photomicrographs of cross-sections of thyroid region of larvae of *Hyla brunnea*.

1. Thyroid anlage with attachment to pharyngeal floor. X 200.
2. Vesicular thyroid anlage after loss of connection with pharynx. X 200.
3. One lobe of thyroid at Stage III. X 600.
4. One lobe of thyroid at Stage IV. X 600.
5. Portion of thyroid at Stage V showing buds (B) and secondary follicles (S.F.). X 600.
6. Portion of thyroid at Stage VI. X 600.
7. Portion of thyroid at Stage VII. X 600.
8. Portion of thyroid of a specimen of Stage VIII with numerous small follicles. X 600.
9. Portion of thyroid of a specimen of Stage VIII with few large follicles. X 400.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walls the epithelial cells seem to have aggregated to form bud-like structures (fig. 4). It would appear that these aggregates of epithelial cells may give rise to new follicles for some small follicles are now present, particularly at the periphery of the gland. Some of them show no stainable colloid, and their epithelium is exclusively of the cuboidal type. The larger follicles, on the other hand, are lined with either cuboidal or columnar cells (fig. 5). Those follicles which have tall columnar cells show the first signs of intracellular vacuolization. The nuclei of these cells are slightly compressed and are basal in position. The colloid is a dense basophilic substance characterized by the presence of many vacuoles which are mostly confined to the peripheries of the colloid masses.

The general structure of the thyroid gland at *Stage VI* is similar to that of the preceding stage. There is, however, an increase in size of the entire gland as well as of the individual follicles. The average value for the volume of the entire gland is 407.0×10^{-4} mm^3 which is the highest value found throughout the stages studied. The histological characteristics of this stage are illustrated in figure 6. Small blood sinuses are scattered between the follicles indicating a moderate amount of vascularization. The follicular walls are made up predominantly of columnar cells with large spherical nuclei which are, in general, located centrally. Mitotic figures are comparatively rare but intracellular vacuoles appear to have increased to a great extent. The staining reaction of the colloid ranges from basophilic to acidophilic. Often both types of staining reaction are present in the same follicle with basophilic colloid surrounding an acidophilic core. Intrafollicular vacuoles, although present, are less abundant than in Stage V.

At *Stage VII* there is a pronounced change in the histology of the thyroid (fig. 7). Often the follicular epithelium is indented, throwing the follicular walls into irregular patterns; the follicles then exhibit various configurations and sizes. The larger ones, in particular, appear much compressed and in some cases collapsed. Frequently, the opposite walls of a follicle approach each other so closely as to be in actual contact. Crowding of the epithelial cells often accompanies the folding of the walls and this may impart a stellate appearance to the follicles as seen in cross-section. In contrast to the preceding stage, the nuclei of the epithelial cells are now elliptical in shape and are so far towards the basal ends of the cells that they al-

most come into direct contact with the cell membranes. The follicular epithelium stains very lightly due to the presence of numerous intracellular vacuoles. Both basophilic and acidophilic colloid are extensively vacuolated. At this stage the average volume of the gland drops to 179.8 x 10^{-4} mm^3 which is about one-half that of the preceding stage.

At *Stage VIII* the histology of the gland has again changed markedly (fig. 8). The follicles seem much distended due to the presence of large amounts of newly secreted colloid. There appears to be a direct relationship between the size of the follicles and the number present in the gland. Thus the thyroid may consist of either many small follicles or a few large ones (figs. 8, 9). The follicular epithelium is predominantly cuboidal and averages less than half as high as that of the preceding stage. The colloid is homogeneous and stains a deep blue. There are relatively few vacuoles; many follicles show none at all. The average value for the volume of the gland has increased to 227.5 x 10^{-4} mm^3.

A number of the anatomical and histological features described in the preceding paragraphs are subject to measurement. Such data are summarized in Table I. The first four horizontal columns give average measurements of body, tail, hind limb, and fore limb for the several stages. The other columns give measurements for various features of thyroid morphology. It will be noted that during the early stages of development (Stages II to IV-B), all measurements show rather regular increases as the thyroid follicles are established and steady growth is carried on. From Stage V-A onward the numerical values of some of the data fluctuate presumably in relation to changes in the physiological activity of the gland.

Significant growth of the hind limbs begins during Stage V-A and is most marked during Stage V-B. It is noteworthy that the volume of the thyroid also increases most markedly at this stage. At Stage V-A the area of the follicular epithelium has increased greatly as compared with Stage IV-B but the colloid area, though it has risen, has not kept pace with the epithelial growth, and the colloid percentage has therefore dropped; during Stage V-B, however, the colloid area shows a sharp rise and the percentage of colloid increases to the maximum value found in this study. These features would seem to indicate increased thyroid activity and colloid storage during the period of hind limb growth.

Stage VI is the stage of fore-limb emergence. The volume of the thyroid now reaches its highest level. There is still further increase in area of the follicular epithelium but the colloid area has dropped as compared with Stage V-B. It thus appears that during fore limb emergence, the secretory activity of the thyroid continues at a high level and a more extensive release of the colloid from the follicles occurs.

The next stage, Stage VII, is characterized by resorption of the tail. At this stage the thyroid gland shows a sharp reduction in volume. The follicular epithelium reaches its highest height at this stage, however, and the colloid percentage continues to decrease, indicating continued high level of secretory activity and colloid release.

At Stage VIII metamorphosis is complete. The thyroid now begins to increase in size but the epithelial height drops markedly. At the same time the colloid percentage shows a great rise. This seems to indicate a new period of rather low secretory activity accompanied by a low rate of release and a resultant storage of the colloid in the follicles.

Discussion

The morphogenesis of the thyroid in *Hyla brunnea* follows the general amphibian pattern as described by Müller (1871), Maurer (1888) and other authors. As in most anurans, the primordium is a solid mass of entodermal cells derived from the pharyngeal floor anterior to the ventral ends of the hyomandibular pouches. The thyroid anlage, like the pharyngeal wall, is heavily pigmented and in the present material the pigment persists until Stage III, at which time follicles are well differentiated, but then the pigment disappears completely. This accords with the observations of Morita (1932) who described the origin of the thyroid in a Japanese species of *Bufo*. However, Müller (1871) reported that pigment material persists in the thyroid tissue of *Rana temporaria* until shortly before tail resorption.

After losing its connection with the pharynx the thyroid anlage migrates posteriorly and then, as in all anurans in which the embryology of the thyroid has been studied, divides into separate lobes. James (1946) has conclusively demonstrated that in *Hyla regilla* this division is attributable to the fact that the thyroid, in its posterior

migration, encounters the "keel" or copula of the hyoid apparatus and splits, one portion passing to either side. By skillful operations during early stages, James was able to produce animals which possessed the second basibranchium but were lacking the copula. Such specimens exhibited single, undivided thyroids. When both basibranchium and copula were missing the thyroid even retained a connection with the pharynx. Judging from the present purely morphological evidence, the hyoid probably plays a similar role in *Hyla brunnea*. The division of the thyroid anlage is observed to occur first at the posterior end where the primordium is in close contact with the copula and for a time the anterior part of the gland remains single so that the organ has the inverted "Y" form which has been observed by D'Angelo and Charipper (1939) and others.

A few tadpoles used in the present study show, in addition to the right and left thyroid lobes a small median lobe. Such accessory thyroids occur quite commonly in some urodeles (Sanders, 1935; Gasche, 1939) and are interpreted as rests left behind during the separation of the thyroid anlage from the pharynx or during its division into lobes. Accessory lobes apparently are not common in anurans however, and indeed D'Angelo and Charipper (1939) say: ". . . in the anura there are no aberrant follicles occurring along the developmental migratory path such as is described for Necturus (Charipper, 1929)".

Previous studies differ in the conclusions drawn concerning the precise time of appearance of a secretion in the developing thyroid. Müller (1871), Hill (1935), and others held that no colloid is produced until after follicles appear; Hirsch (1928) claimed that colloid secretion occurs coincidentally with follicle formation; but most investigators (Sklower, 1925; Uhlenhuth, 1927; Sanders, 1935; Gasche, 1939) have maintained that both intracellular and extracellular colloid droplets are demonstrable before any organized follicles are present. Concerning this, nothing definite can be concluded from the findings of the present study since excessive pigment material masks the finer details of the glands at the stages preceding follicle differentiation. It is clear, however, that the first colloid formed is chromophobic. When primary follicles are first observable most of the cell groups surround clear spaces while some enclose a granulated substance which stains only very faintly. The chromophobic nature of the first secretion substance has been reported for

a number of vertebrates including frog (Kerr, 1939), lizard (Eggert, 1934), turtle (Dimond, 1952), chick (Venzke, 1949), and rabbit (Togary et al. 1952).

Various hypotheses have been advanced concerning the way in which follicles first form and later increase or decrease in number. In *Hyla brunnea* the thyroid anlage is in the form of strings or cords of cells immediately before follicle formation and when follicles are first seen they present an appearance which seems consistent with the idea that they originate by constriction of balls of cells along these cords. These early follicles do not show any enclosing connective tissue and there is, therefore, no evidence that connective tissue penetration as described by Müller (1871), Sklower (1925), and Gasche (1939), plays a major role in the differentiation of primary follicles. So-called secondary follicles have been described as arising by either fusion or division of pre-existing follicles (Klöse, 1931; Sanders, 1935). Sugiyama (1941), who has described the process of follicle formation in the albino rat and mouse in some detail, says that most secondary follicles originate from local thickenings of the walls of primary follicles. These thickenings increase in size by proliferation of new cells until solid buds are formed which project into the stroma of the gland. The buds then separate from the parent follicles, acquire lumina, and gradually increase in size. Sugiyama also described secondary follicles arising from "collapsed" primary follicles. This is said to occur after mature follicles have discharged their colloid, the cells of some parts of the walls then rearranging themselves to form new follicular entities. In *Hyla brunnea* there is rather convincing evidence of formation of secondary follicles by budding (see fig. 5). Such buds were observed as early as Stage III but were most abundant at Stage V. Formation of secondary follicles from "collapsed" follicles may possibly be indicated at Stage VII but evidence for this is by no means unequivocal. At none of the stages was there any indication of origin of secondary follicles by fusion of primary ones.

The chief point of interest in the present study has been an attempt to ascertain the relation between the secretory activity of the thyroid and the metamorphoric pattern in *Hyla brunnea*. A number of criteria for judging the relative activity of the thyroid are in general use. These include changes in the relative volume of the gland, the height of the follicular epithelium, the amount of intra-follicular col-

loid, the extent of vacuolization of the colloid and the abundance of intracellular vacuoles and colloid droplets. Although there have been some differences of opinion concerning the details of some of these features (see Lynn and Wachowski, 1951), when all are taken together, they form a reliable index of whether the gland is in an actively secretory phase or a relatively inactive quiescent phase and of whether the material secreted is being rapidly released into the blood stream or is mostly being stored in the gland. A highly active gland exhibits a high columnar epithelium with many intracellular vacuoles and colloid droplets; an inactive gland has a cuboidal or squamous epithelium with no vacuoles or colloid droplets. The colloid within the follicular lumen is usually basophilic in a highly active gland and may show many nonstaining vacuoles, especially around the periphery of the colloid mass. The colloid of an inactive thyroid is usually acidophilic and without vacuoles. There is evidence, however, that the staining reaction of the colloid may be more directly related to phases of storage and release of secretion than to the actual secretory activity of the gland. Acidophilic colloid probably represents material which has been stored for some time while basophilic colloid is newly formed. Rapid release of stored secretion is, of course, indicated by a sharp decrease in the amount of intrafollicular colloid and consequent collapse of the follicle walls.

Beginning with the studies of Allen (1919) on *Bufo* and Hoskins and Hoskins (1919) on *Rana sylvatica* a number of investigations have been carried out concerning the structure of the thyroid gland before, during, and after metamorphosis in various anurans. These studies indicate that the thyroid begins a significant increase in size and secretory activity at about the time when hind limbs begin to grow. As hind limb growth continues, the activity of the gland also continues to increase and reaches a maximum near the time when the tail is undergoing resorption. After this the secretory activity, as indicated by epithelial height and vacuolization, decreases to a relatively low level. A rapid decrease in colloid and a collapse of follicular walls has been reported to occur at the climax of metamorphosis in *Rana temporaria* (Sklower, 1925) and *Rana pipiens* (D'Angelo and Charipper, 1939) but Etkin's studies (1930, 1936) on several species of *Rana* and on *Pseudacris triseriata* indicate that there is only a gradual regression with no distinct stage of follicular evacuation.

The present findings for *Hyla brunnea* are in agreement with the general results outlined above. The volume of the gland increases steadily to Stage VI, the time of fore limb emergence, drops markedly at the period of tail resorption (Stage VII), and then shows a slight increase after metamorphosis has been completed (Stage VIII).

The changes in epithelial height and in vacuolization indicate a high rate of secretory activity beginning during Stage IV and continuing through Stage VIII. The staining reaction of the colloid and the volume of the colloid, as indicated by the area occupied in a median section of the thyroid, indicate that colloid storage predominates through Stage VI and is most marked during Stage VIII.

The significance of the changes in the thyroid function in relation to the metamorphic process has been subjected to extensive investigations by Etkin (1930, 1932, 1935, 1936). His work, which includes both morphological and physiological studies, indicates that the increased growth and activity of the thyroid during early metamorphosis (period of rapid hind limb growth), though it results in accumulation of stored colloid in the gland, is also accompanied by a gradual rise in the concentration of the thyroid hormone in the blood and tissues. This gradual rise is responsible for the events of metamorphic climax (fore limb emergence, loss of chitinous teeth, resorption of tail, etc.). Following this, a regression occurs but, as stated above, Etkin was unable to find evidence in his material of a well-defined "release phase" characterized by a quick emptying of the thyroid follicles. Aleschin (1935, 1936) maintained that, in *Rana pipiens,* the thyroid gland shows histological evidence of being in an inactive state during the period of tail resorption and therefore concluded that the processes of late metamorphic climax, once they are initiated, continue automatically without any dependence upon the thyroid. This idea is not supported by Etkin's work or by the studies of Clements (1932), D'Angelo and Charipper (1939) and others. In *Hyla brunnea* the history of the thyroid during pre-metamorphosis and during metamorphosis itself seems to be in complete agreement with that described by Etkin for *Rana* and *Pseudacris*. It appears, however, that *Hyla brunnea* does show a stage of sudden colloid release at Stage VII with an extensive evacuation of colloid quite similar to that described by Uhlenhuth (1927) for *Ambystoma*. This release phase corresponds with the period of metamorphic climax. Possibly it results in a greater rapidity of tail resorption, intestinal short-

ening, gill degeneration, etc., than occurs in *Rana* and *Pseudacris*, however, the rate at which these changes occur could not be studied in the present material since the precise age of most specimens was not known. Since the thyroid shows evidence of intense secretory activity as well as colloid release during tail resorption, it is clear that the conditions in *Hyla brunnea* do not agree with Aleschin's hypothesis that the events of late metamorphosis are independent of thyroid functioning.

Since the general features of the metamorphic pattern in *Hyla brunnea* agree so closely with those found in *Rana, Bufo* and *Pseudacris* and since the morphological changes in the thyroid gland seem to be correlated with metamorphic events in much the same way as it is in these genera, one must conclude that despite the modifications exhibited by *Hyla brunnea* in relation to its unusual habitat, the mechanism of thyroid control of metamorphosis is not greatly modified. This is in striking contrast to the profound modifications seen in *Eleutherodactylus* (Lynn and Peadon, 1955), a genus in which most metamorphic changes appear to be completely emancipated from dependence upon thyroid stimulation.

Summary

1. In *Hyla brunnea* the thyroid anlage originates at the late neurula stage as a solid proliferation of entodermal cells from the pharyngeal floor.

2. The posterior migration of the thyroid anlage, its division into two separate lobes, and the differentiation of primary follicles occur in a manner which agrees closely with that described for other anurans such as *Rana* and *Bufo*.

3. The origin of secondary follicles seems to occur mainly, perhaps exclusively, by the process of budding from primary follicles.

4. Study of the histological changes in the thyroid during stages subsequent to primary follicle formation indicates that the secretory activity of the gland begins to increase shortly before rapid growth of the hind-limbs is initiated and continues at a high level to the period of rapid tail resorption.

5. Indications of sudden release of colloid are noted in correlation with metamorphic climax.

6. After metamorphosis the secretory activity of the gland declines sharply and storage of the colloid predominates.

References

ALESCHIN, B. 1935. Métamorphose des amphibiens comme effet morphogénétique de la glande thyroïde. *Bull. Histol. appl.*, **12**, 5-28.

————. 1936. Die Schilddrüse in der Entwicklung und in der Metamorphose von *Rana temporaria*. *Acta Zool.*, **17**, 1-54.

ALLEN, B. M. 1919. The development of the thyroid glands of Bufo and their normal relation to metamorphosis. *J. Morph.*, **32**, 489-507.

BRINK, H. E. 1936. Die Skildlier en Metamorphose bij die Amphibia. Ann. Univ. Stellenbosch, **14**, 1-111.

————. 1939. A histological and cytological investigation of the thyroids of *Arthroleptella bicolor villiersi* and *Bufo angusticeps* during the normal and accelerated metamorphosis. *Proc. Linn. Soc. Lond.*, **151**, 120-125.

CHARIPPER, H. A. 1929. Studies on amphibian endocrines. I. The thyroid gland of *Necturus maculosus*. *Anat. Rec.*, **44**, 117-141.

CLEMENTS, D. I. 1932. Comparative histological studies of the thyroids and pituitaries in frog tadpoles in normal and accelerated metamorphosis. *J. Roy. micr. Soc.*, **52**, 138-148.

D'ANGELO, S. A. 1941. An analysis of the morphology of the pituitary and thyroid glands in amphibian metamorphosis. *Amer. J. Anat.*, **69**, 407-437.

————, & CHARIPPER, H. A. 1939. The morphology of the thyroid gland in the metamorphosing *Rana pipiens*. *J. Morph.*, **64**, 355-371.

DIMOND, SR. M. T. 1952. The embryology of the thyroid gland in the snapping turtle, *Chelydra serpentina serpentina*. Master's Diss., Catholic U.

EGGERT, B. 1934. Die embryonale Differenzierung der Schilddrüse und des ultimobranchialen Körpers von *Lacerta agilis* L. and *Lacerta vivipara* Jacq. *Z. Wiss. Zool.*, **145**, 687-710.

ETKIN, W. 1930. Growth of the thyroid gland in *Rana pipiens* in relation to metamorphosis. *Biol. Bull.*, **59**, 285-292.

————. 1932. Growth and resorption phenomena in anuran metamorphosis I. *Physiol. Zool.*, **5**, 275-300.

————. 1935. The mechanism of anuran metamorphosis. I. Thyroxine concentration and the metamorphic pattern. *J. Exp. Zool.*, **71**, 317-340.

————. 1936. The phenomena of anuran metamorphosis. III. The development of the thyroid gland. *J. Morph.*, **59**, 69-89.

GASCHE, P. 1939. Beitrag zur Entwicklung von *Salamandra salamandra* L. mit besonderer Berücksichtigung der Winterphase, der Metamorphose und des Verhaltens der Schilddrüse. *Rev. Suisse Zool.*, **46**, 403-458.

GUDERNATSCH, J. F. 1912. Feeding experiments on tadpoles. I. The influence of specific organs given as food on growth and differentiation. A contribution to the knowledge of organs with internal secretion. *Arch. Entw. Mech. Org.*, **35**, 457-483.

HILL, B. H. 1935. The early development of the thyroid gland in *Amia calva*. *J. Morph.*, **57**, 533-545.

HIRSCH, G. 1928. Metamorphose, Brunst, Neotenie und Schilddrüse bei *Triton taeniatus*. *Microkosmos*, **22**, 65-70.

HOSKINS, E. R., & HOSKINS, M. M. 1919. Growth and development of amphibia as affected by thyroidectomy. *Jour. Exp. Zool.*, **29**, 1-69.

JAMES, M. S. 1946. The role of the basibranchial cartilages in the early development of the thyroid of *Hyla regilla*. *U. Calif. Publ. Zool.*, **51**, 215-228.

KERR, T. 1939. On the histology of the developing pituitary in the frog (*Rana temporaria*) and in the toad (*Bufo bufo*). *Proc. Zool. Soc. Lond.*, **109**, 167-180.

KLÖSE, W. 1931. Beiträge zur Morphologie und Histologie der Schilddrüse, der Thymusdrüse und des postbranchialen Körpers von *Proteus anguineus*. *Z. Zellforsch.*, **14**, 385-439.

LYNN, W. G. 1936. A study of the thyroid in embryos of *Eleutherodactylus nubicola*. *Anat. Rec.*, **64**, 525-539.

————. 1942. The embryology of *Eleutherodactylus nubicola*, an anuran which has no tadpole stage. *Carnegie Cont. Embryol.*, **30**, 27-62.

————, & PEADON, A. M. 1955. The role of the thyroid gland in direct development in the anuran, *Eleutherodactylus martinicensis*. GROWTH, **19**, 263-286.

————, & WACHOWSKI, H. E. 1951. The thyroid gland and its functions in cold-blooded vertebrates. *Quart. Rev. Biol.*, **26**, 123-168.

MAURER, F. 1888. Schilddrüse, Thymus und Kiemenreste der Amphibien. *Morph. Jb.*, **13**, 296-382.

MORITA, S. 1932. Quantitative Untersuchungen über die Schilddrüse von *Bufo* in Beziehung zur Metamorphose. *J. Sci. Hiroshima U.*, **2**, 1-20.

MÜLLER, W. 1871. Über die Entwicklung der Schilddrüse. *Jena Z. Med. Naturw.*, **6**, 428-453.

POLLISTER, A. W., & MOORE, J. A. 1937. Tables for the normal development of *Rana sylvatica*. *Anat. Rec.*, **68**, 489-496.

SANDERS, J. M. 1935. The development of the thyroid gland in urodeles. *J. Morph.*, **57**, 597-615.

SKLOWER, A. 1925. Das incretorische System im Lebenscyclus der Frösche (*Rana temporaria* L.) I. Schilddrüse, Hypophyse, Thymus und Kiemendrüsen. *Z. vgl. Physiol.*, **2**, 474-523.

STEINMETZ, C. H. 1952. Thyroid function as related to the growth of tadpoles before metamorphosis. *Endocrinol.*, **51**, 154-156.

————. 1954. Some effects of thyroxine and antithyroid compounds on tadpoles and their relation to hormonal control of growth. *Physiol. Zool.*, **27**, 28-40.

SUGIYAMA, S. 1941. The embryonic development of the thyroid gland in the albino rat and the mouse with special emphasis on its histogenesis. *Fol. Anat. Jap.*, **20**, 465-506.

TOGARI, CH., SUGIYAMA, SH., & SAWASAKI, Y. 1952. On the prenatal histogenesis of the thyroid gland of the Rabbit with special emphasis on the histometrical measurements. *Anat. Rec.*, **114**, 213-229.

UHLENHUTH, E. 1927. Die Morphologie und Physiologie der Salamander-Schilddrüse. I. Histologischembryologische Untersuchungen des Sekretionsprozesses in den verschiedenen Lebensperioden der Schilddrüse des Marmorsalamanders, *Ambystoma opacum*. *Arch. Entw. Mech. Org.*, **109**, 611-749.

————. 1938. A quantitative approach to the secretion process of the thyroid. *The Collecting Net*, **13**, 1-8.

VENZKE, W. G. 1949. Morphogenesis of the thyroid gland of chicken embryos. *Am. J. Vet. Res.*, **10**, 272-281.

Growth, 1956, **20**, 315-319.

EXPERIMENTAL PRODUCTION OF LYMPHOCYTIC FOCI IN THE THYROID

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In 16 per cent of human thyroids obtained by autopsy (5), lymphocytic foci are present. There is no agreement in the literature about their significance. Some workers (9, 4) regard them as normal findings, others (3, 7) as manifestation of a thymolymphatic constitution, and a third group (11, 13, 12) of investigators explain them as focal areas of thyroiditis.

If the third view is correct, it should be possible to produce these lymphocytic foci experimentally.

Material and Methods

Fifteen adult male guinea pigs (1180 to 1400 grams) were used, and were fed a non-goitrogenic well balanced diet (Purina chow and lettuce) with tap water. Each experimental animal received one intraperitoneal injection of 100 microcuries of radio-active iodine. The animals were killed 14 days after injection. Their thyroids were measured, weighed and fixed in formalin, dehydrated and embedded in paraffin. The paraffin sections, 5 microns thick, were stained with hematoxylin and eosin.

Results

Histologic study of thyroids from many adult guinea pigs fed the same diet and kept in the same room as the experimental animals never revealed lymphocytic foci. On the other hand, every one of the 15 guinea pigs which had received an injection of 100 microcuries of radio-active iodine showed aggregates of lymphocytes.

These lymphocytic foci were situated in the interfollicular stroma of both thyroid lobes and consisted of from 10 to over 100 round cells. In these glands macrophages in the lumen of adjacent follicles were always present. The epithelial wall of these follicles was intact. Their

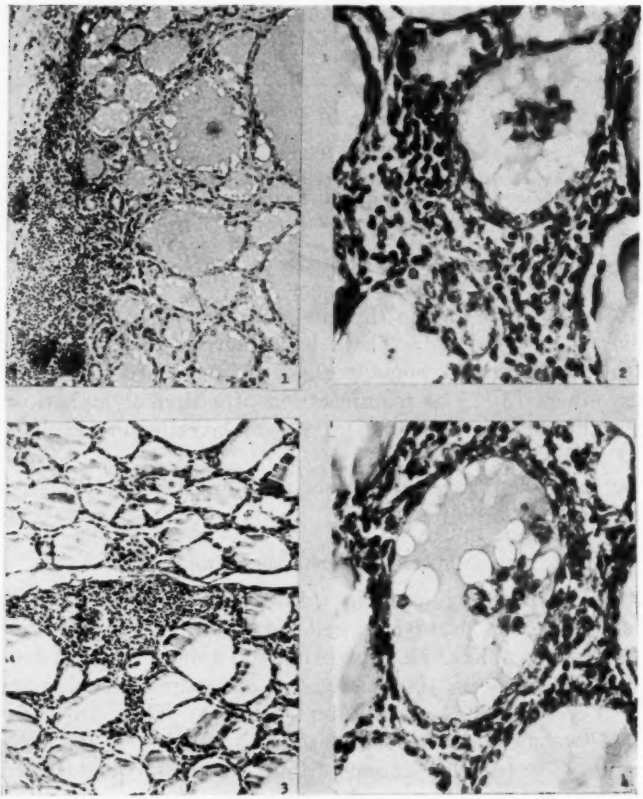

FIGURE 1

Lymphocytic focus in thyroid of 66 year old female who died from hemorrhagic glomerulonephritis.

FIGURE 2

High magnification of the same thyroid. Follicle adjacent to lymphocytic focus contains macrophages. The colloid is vacuolated (cellular resorption).

FIGURE 3

Lymphocytic focus in thyroid of guinea pig 10 days after injection of I^{131}.

FIGURE 4

High magnification of same thyroid. In a follicle with intact on wall macrophages are present and the colloid is vacuolated. Lymphocytes are infiltrating the adjacent stroma.

colloid showed vacuoles around the macrophages and appeared in a state of resorption. Occasionally macrophages were noticed in intercellular spaces of the follicle wall and in the stroma. Droplets of lipid could be demonstrated in the macrophages with Sudan black stain.

The triad, in the thyroid, of macrophages, cellular resorption of colloid, and accumulation of lymphocytes, occurred in every guinea pig following injection of 100 microcuries of I^{131}. Our results sup-

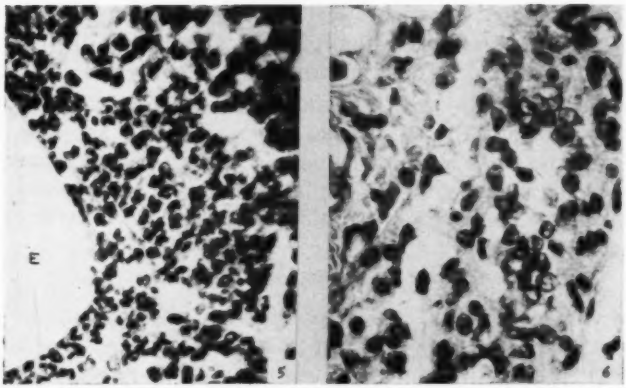

FIGURE 5

Acute inflammation of subcutaneous tissue of guinea pig 48 hours after injection of lipid extract (E) of thyroid colloid.

FIGURE 6

Aggregate of monocytes and lymphocytes in subcutaneous tissue of guinea pig, 6 days after injection of lipid extract.

port the view held by Simmonds, v. Werdt and Wegelin that lymphocytic foci in non-goitrous thyroids are inflammatory reactions and not manifestations of a thymolymphatic constitution.

The association of lymphocytes in the stroma with intrafollicular macrophages was constant in our experimental animals as it is in human thyroids.

From the experimental studies of Dobyns (1) and Schwartz (10) it is known that excess of TSH increases the lipid content of thyroid cells and of the colloid. Since we could demonstrate lipid droplets in the macrophages of our experimental animals we believe that these

macrophages have been attracted into thyroid follicles by a lipid-rich colloid. These cells ingest the altered colloid, return to the stroma where some of them disintegrate. When the colloid thus becomes liberated, it acts as a chemical irritant on the vascular stroma. Ferguson (2), ourselves (6), and recently Kracht (8), have produced severe inflammation in the subcutaneous tissue of guinea pigs after injection of lipid extracts from thyroid colloid.

In our experiments, the radio-active iodine, while not producing visible necrosis, apparently damages the thyroid epithelium enough to decrease thyroid function. The thyroid deficiency stimulates the pituitary to increased production of TSH, the lipid in the colloid becomes increased and attracts macrophages into thyroid follicles.

Since in human thyroids lymphocytic foci most frequently occur after 50 years of age, low thyroid function due to age may be the cause of increased TSH production. In the experimental animal, as well as in the human thyroid, the ultimate cause of lymphocytic foci is in our opinion a chemical irritation of interfollicular tissue by lipid-rich colloid.

Summary and Conclusions

1. Lymphocytic foci can be produced in the thyroids of guinea pigs with one injection of 100 microcuries of radio-active iodine.

2. Adjacent to these lymphocytic foci macrophages were always found in the lumen of thyroid follicles.

3. Our experiments favor the view that lymphocytic foci in nongoitrous thyroids represent an inflammatory process. The underlying cause is probably a chemical irritation by lipid-rich colloid carried by macrophages into the interfollicular stroma.

References

1. Dobyns, B. M. 1946. Studies on exophthalmos produced by thyrotropic hormone. *Surg., Gynec. & Obst.*, **82**, 717-722.
2. Ferguson, J. A. 1933. Tissue reaction to colloids and lipoids from the human thyroid gland. *Arch. Path.*, **15**, 244-254.
3. v. Gierke, E. Quoted by Simmonds (11).
4. Ham, Arthur W. 1953. Histology. Philadelphia: Lippincott. Ed. 2, pp. 756.
5. Hellwig, C. A. 1954. Colloidophagy in the thyroid gland. *A. M. A. Arch. Path.*, **58**, 151-152.
6. Hellwig, C. A., & Wilkinson, P. N. 1956. Experimental production of thyroiditis. *A. M. A. Arch. Path.*, **62**, 23-29.
7. Himmelberger, L. R. 1932. The significance of lymphatic tissue and adenoma-like areas in the thyroid gland. *Ann. Int. Med.*, **5**, 1404-1420.

8. KRACHT, JOACHIM. 1955. Thyrotrophin and experimental thyroiditis. *Acta endocrinal.*, **18**, 437-444.
9. MAXIMOW, ALEXANDER A. 1936. Textbook of Histology. Philadelphia: Saunders. Ed. 2, pp. 662.
10. MORTON, M. D., & SCHWARZ, J. R. 1953. The stimulation in vitro of phospholipid synthesis in thyroid tissue by thyrotropic hormone. *Science*, **117**, 103-104.
11. SIMMONDS, M. 1913. Ueber lymphatische Herde in der Schilddrüse. *Virchow's Arch. f. path. Anat.*, **211**, 73-89.
12. WEGELIN, C. 1949. Die Entzündungen der Schilddrüse. Aertzl. Monatshelft. f. d. beruf. Fortbild., 1-43.
13. v. WERDT, F. 1911. Ueber Lymphfollikelbildung in Strumen. Frankfurt *Ztschr. f. Path.*, **8**, 401.

Growth, 1956, **20**, 321-322.

BOOK REVIEW

The American Arbacia and Other Sea Urchins. Ethel Browne Harvey. June, 1956. Princeton University Press. 298 pages plus 16 plates. $6.00.

Contrary to the implication of its title, this book is primarily concerned with work in experimental embryology and cellular physiology. Investigators in these fields will find it both interesting and useful. It may be criticized by some for its inconsistency of style, pace, format, and purpose. It is, however, just the book which Ethel Harvey should have written and the varied styles are appropriate to the several purposes achieved.

It should be recalled that except for amphibian and chicken eggs no embryological object has been used so much and by so many investigators as the sea urchin egg. In America almost all of the early work and much of the recent work has been at Woods Hole where *Arbacia* is available. Quoting the author: "We have a fine heritage of experimental research on the *Arbacia* egg, and it is partly in an endeavor to gather together this work and make it more readily available to later investigators that this book has been written." For about thirty years Dr. Harvey has devoted herself to studies of these eggs. As younger investigators with new problems and new methods have attempted to use *Arbacia* eggs they have sought and received her aid in learning how to work with this unfamiliar organism. Her book now describes fully the "tricks of the trade".

One impediment to experimental work on *Arbacia* has been the failure of anyone to take the time to work out and publish an adequate description of its normal embryology. This is now done with extensive photographic illustration in the second part of the book. The addition of some diagrams might have been helpful here; maps showing prospective fates of cells or regions of blastula and gastrula, and diagrams of the internal structure of the pluteus would have been useful. Perhaps for *Arbacia* the necessary observations have not been completed.

The third and briefest section of the book deals with experiments and methods of centrifugation, the field in which the author has made the most conspicuous contributions.

The fourth part is a compilation of experimental work arranged alphabetically by subjects. The comprehensiveness of this section

of 85 pages is suggested by the more than 60 topics treated: Ageing of eggs, Amoebocytes, Amoeboid eggs, Anaesthetics, Calcium, . . . Vitelline membrane, X-rays, and Yolk Granules. Under each topic are many subtopics with statements of experimental results. The author does not attempt to evaluate the several thousand results cited. It is this section which will be of greatest interest and practical use to investigators in cellular physiology, whether or not they have any concern with embryological problems. The reader of this review is advised to look up his own favorite subject in this section; I think this effort will be repaid.

The bibliography contains about 1500 items and is essentially complete for *Arbacia punctulata*. There is an index.

The first section of the book, some 67 pages, is appropirate to the title: The American Arbacia and Other Sea Urchins. It is good natural history in a satisfactory literary style with extensive documentation. Anyone likely to read this review will find this part of the book enjoyable, though not required, reading.

Altogether the book is a thorough compilation. All who work with sea urchin eggs will need it as a reference; and its value for physiologists has already been explained. It is not a synthesis, summary, review, nor history of those problems of development in which sea urchins have been used. The facts have been assembled; their interpretation and significance lie outside the scope and purposes of the book.

SEARS CROWELL